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Free Radical Toxicology

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Free-Radical Defense and Repair Mechanisms

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INTRODUCTION

Defense and repair systems are critical modulators of cellular oxidative damage. In this chapter, we discuss the functions and interplay of antioxidants and antioxidant enzymes and emphasize the complementary nature of these systems. To explain the need for such diverse antioxidant defense and repair systems, we describe pertinent aspects of the formation and chemistry of biologically relevant oxidants. We also discuss some aspects of free-radical toxicity that is associated with biotransformation of various chemicals. In doing so, we emphasize those oxidants against which cellular antioxidant defense is directed and perhaps account for Nature's selection of specific antioxidant defenses. Detailed discussions of the formation and chemistry of reactive oxidants are presented elsewhere in this volume.

The continual formation of reactive oxygen species is a physiological necessity and an unavoidable consequence of oxygen metabolism. However, when generated in excess, they can be toxic, particularly in the presence of transition metal ions such as iron or copper (elsewhere in this volume); for a review see Halliwell and Gutteridge (1). Since defense systems are present and functioning under normal conditions, endogenous free radicals do not necessarily place biological tissues and cells at risk. However, these defense systems can be overwhelmed during various pathological conditions caused by xenobiotics, anoxia, radiation, and loss of extracellular calcium. Excess generation of free radicals within tissues can cause damage to vital cellular constituents.

It is estimated that nearly 90% of the total O_2 consumed by mammalian species is delivered to mitochondria, where a four-electron reduction to H_2O by the respiratory chain is coupled to ATP synthesis (2,3). Nearly 4% of mitochondrial O_2 is incompletely reduced by leakage of electrons along the respiratory chain, especially at ubiquinone, forming ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen, and hydroxyl radical (HO^\bullet) (2,3). Richter (4) calculated that during normal metabolism, one rat liver mitochondrion produces 3×10^7 superoxide radicals per day. It is estimated that superoxide and hydrogen peroxide steady state concentrations are in the picomolar and nanomolar range, respectively (5). Jones et al. (6) have estimated the hepatocyte steady state H_2O_2 concentration to be up to 25 μM . These events are thought to contribute over 85% of the free-radical production in mammalian species. Sohal (7) has concluded that there is a variation in the sites of superoxide or hydrogen peroxide generation among mitochondria from different tissues and species. This is a result of the rate of mitochondrial superoxide production is dependent on at least three variables: (1) ambient oxygen concentration; (2) levels of autoxidizable respiratory carriers, especially ubiquinone; and (3) the redox state of the autoxidizable carriers (2,5,7-9).

Toxic chemicals can cause oxidant formation through several mechanisms. In some instances, metabolism drives the formation of oxygen-containing reactive intermediates through a process known as redox cycling. Many other chemicals can also undergo bioactivation to form biological reactive intermediates that bind to macromolecules and indirectly enhance the formation of oxygen radicals. Some chemicals induce inflammatory responses, in which release of reactive oxygen and nitrogen radicals by stimulated phagocytes constitutes an oxidative challenge. Still other chemicals undergo facile photoexcitation reactions that lead to the formation of either singlet oxygen, free radicals, or both. Oxidant generation through any of these scenarios may lead to the oxidation of critical functional groups on macromolecules, to peroxidation of lipids, and to oxidation of other susceptible cellular constituents.

The evolution of bioactivation processes that form biological reactive intermediates, both free radical and ionic, probably necessitated the concomitant evolution of cellular defense and repair systems for cell survival. All tissues and cells contain defense systems for detoxification of biological reactive intermediates and to prevent or limit cellular damage. Toxic processes have reversible and irreversible features that are a consequence of the interplay with cellular defense and repair systems. Reversible toxicity occurs even with chemicals known as "safe" chemicals. Dose determines whether any chemical causes irreversible toxicity. Irreversible toxicity may cause cell death regardless of what antidotal or preventive measures are taken after exposure of cell or tissues to a sufficient dose of the toxic chemical. Death occurs when loss of cellular integrity occurs to such a degree that free exchange between the intracellular constituents and the surrounding milieu prevents cell survival. We review here the mechanisms by which cells are protected by defense and repair systems to prevent or limit cellular damage and death.

THE INITIATION AND PROPAGATION OF OXIDATIVE DAMAGE

In this section we describe general characteristics of the origin of free radicals and related oxidants. Rather than provide a comprehensive documentation of the sources of oxidants, we focus on how the common primordial oxidants superoxide and nitric oxide lead to the formation of a number of more reactive oxidants. We also distinguish between the initiation and propagation of oxidative damage and consider the roles of these processes in oxidative injury. This establishes a framework for describing the interlocking, complementary nature of cellular antioxidant defense.

Superoxide, Nitric Oxide, and Transition Metals as Primordial Oxidant Sources

Major contributors to oxidative injury are (1) xenobiotics or endogenous factors that can cause increased superoxide generation, (2) factors that stimulate the production of nitric oxide, and (3) processes that disrupt heme proteins or other metalloproteins to enhance the contribution of transition metals to oxidant generation. Increased superoxide generation can result from agents that stimulate phagocytic cells including polymorphonuclear leukocytes and macrophages, which generate large quantities of superoxide as a mechanism for destruction of foreign cells. Free-radical generation by neutrophils can be as high as 200 nmol/ 10^6 cells/h (10,11). It is known that an NADPH oxidase provides the catalysis for the rapid consumption of molecular oxygen (12,13). Even nonphagocytic cells can be stimulated by agents such as phorbol esters to increase superoxide production through the conversion of xanthine dehydrogenase to xanthine oxidase (14). Chemicals also can induce superoxide formation by catalyzing electron transfer from cellular redox proteins to molecular oxygen (redox cycling), as discussed elsewhere in this volume.

Many of the same stimuli that induce macrophages to produce superoxide also stimulate the production of nitric oxide by inducible nitric oxide synthetase (see Morris and Billiar, 15, for a recent review). Nitric oxide produced in relatively high yield by these inducible enzymes may react with oxygen or superoxide to yield more reactive oxidants, as described later. Finally, oxidants, nonoxidizing reactive intermediates, and other mediators of cellular injury may disrupt hemoproteins and other metalloproteins to release transition metal ions, particularly iron and copper, which may amplify damage by catalyzing the formation of highly reactive radicals. Reactions of superoxide, nitric oxide, and metals lead to oxidative injury by forming secondary oxidants that are believed to be responsible for actually causing most biological oxidative damage. These reactions and some of their intermediates are key control points in the initiation and propagation of oxidative damage. These critical reactions and intermediates also are often the targets of antioxidant defense.

Reactions of Superoxide, Nitric Oxide, and Metals: Formation of Secondary Oxidants

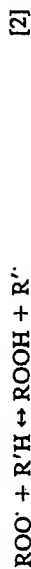
Superoxide is itself a reasonably strong oxidant ($E^\circ = 940 \text{ mV at pH } 7$) (16), but most of its pro-oxidant chemistry is thought to be due to formation of its conjugate acid HOO^\cdot ($\text{pK}_a 4.9$), which may initiate lipid peroxidation by hydrogen abstraction from hydroperoxides. Superoxide decomposition gives rise to even more reactive oxidants. Enzyme-catalyzed and nonenzymatic dismutation yield hydrogen peroxide, a nonradical pro-oxidant that may freely diffuse across membranes. Metal-catalyzed cleavage of hydrogen peroxide (the Fenton reaction) forms hydroxyl radical or similarly reactive high-valent metal-oxo complexes, which are the most reactive oxidants known in biological systems (Koppenol, chapter 1, this volume).

Superoxide also may react at diffusion limited rates with nitric oxide to form the highly reactive nonradical peroxynitrite (17). Peroxynitrite is an excellent oxidant for biological thiols, and its conjugate acid peroxynitrous acid forms a reactive oxidant with reactivity similar to hydroxyl radical (18). In addition to its reaction with superoxide, nitric oxide also may autoxidize to nitrogen dioxide, which is a highly reactive initiator of free-radical reactions (19).

Transition metal ions may greatly enhance oxidative damage in two general ways. First, they may reductively cleave hydrogen peroxide and alkyl hydroperoxides to hydroxyl and alkoxy radicals, respectively (i.e., the Fenton reaction, reviewed by Koppenol, chapter 1, this volume). The extent to which this reaction occurs *in vivo* depends on the availability of metal ions. However, in all but the most stringently demetalized experimental systems, metal-catalyzed cleavage of lipid hydroperoxides probably is the driving force for most lipid peroxidation studied *in vitro* (20–22). Metal-catalyzed cleavage of lipid hydroperoxides greatly amplifies lipid peroxidation, and analogous reactions may contribute significantly to the autoxidation of proteins and DNA as well. Metals also may directly oxidize thiols to thyl radicals, which may add oxygen to form more highly oxidizing intermediates (reviewed in refs. 16 and 23). The reduced forms of the metal ions formed by these reactions may participate in Fenton chemistry as described earlier.

Formation and Reactions of Peroxyl Radicals: Propagation of Oxidative Damage

The reactive intermediates discussed earlier all contribute to oxidative damage either by direct reaction with oxidizable biomolecules (hydroxyl radical, alkoxy radical, peroxynitrite, hydroperoxyl radical, nitrogen dioxide) or by serving as immediate precursors to radicals (hydrogen peroxide, organic hydroperoxides, peroxynitrous acid). The radical species react with lipids, proteins, DNA or other biomolecules either by addition, hydrogen abstraction, or electron transfer to form (primarily) biomolecule-derived, carbon-centered radicals. In aerobic environments, these carbon centered radicals reversibly add oxygen to form peroxyl radicals, which then react with adjacent biomolecules:



This process is termed *propagation* and is normally considered in terms of lipid peroxidation (see Sevanian and McLeod, chapter 4, this volume). However, the same sequence of reactions also can contribute to the spread of oxidative damage in proteins, DNA, and other biomolecules. A free-radical chain initiated by a single hydroxyl radical in a lipid membrane thus may lead to over 20 propagation cycles before the chain is terminated (24). All of the other radicals discussed earlier can initiate radical chains, which then are propagated in the same manner. It is not surprising in this context that peroxyl radical propagation reactions can contribute the bulk of oxidative damage regardless of the specific initiating oxidants involved.

THE ORGANIZATION OF ANTIOXIDANT DEFENSE

Cellular antioxidant defenses are organized into several tiers against oxidant challenges. The juxtaposition of oxidant challenge and antioxidant defense is illustrated in Figure 1. For clarity, we have greatly simplified the highly complex chemistry involved in the initiation and propagation of oxidative damage. Key elements of oxidative challenges are described earlier and depicted in Figure 1. These include (1) the formation of primordial radicals superoxide and nitric oxide, (2) the generation of highly reactive secondary intermediates (e.g., hydroxyl and hydroperoxyl radical, peroxynitrite), which may initiate free-radical chain reactions, and (3) amplification by peroxyl radical dependent chain propagation. Another key element of oxidant challenge is the role of transition metals both in forming highly reactive secondary intermediates (e.g., Fenton chemistry) and in amplifying the propagation of oxidative damage.

Antioxidant defenses are directed against several aspects of the oxidant challenge. A general observation is that the enzyme-mediated antioxidant defenses are directed against the primordial initiator superoxide and the less reactive secondary mediators hydrogen peroxide and organic hydroperoxides. Small-molecule chain-breaking antioxidants are instead directed primarily against peroxyl radicals involved in radical propagation. Cellular antioxidant defenses thus serve complementary functions within the context of a multitiered oxidant challenge. Characteristics of the individual components of cellular antioxidant defense are described later. More extensive discussion of the antioxidant roles of glutathione, vitamin E, and ascorbate is provided in succeeding sections.

Superoxide Dismutases, Catalase and Glutathione Peroxidases

Superoxide dismutases (SOD) catalyze the dismutation of superoxide to oxygen and hydrogen peroxide. A major protective benefit is derived from enzymatic

in cytosol and mitochondria and reduces hydrogen peroxide and some organic hydroperoxides to water and alcohols, respectively. A similar enzyme found in extracellular fluids and in plasma shares sequence homology with the intracellular enzyme, but is a separate gene product (32). Another enzyme, phospholipid hydroperoxide glutathione peroxidase, is a monomeric protein containing one selenocysteine (33). This enzyme reduces phospholipid hydroperoxides to the corresponding alcohols.

Ursini et al. (33) has purified and characterized an interfacial glutathione peroxidase. This enzyme has been shown to reduce linoleic acid hydroperoxides, cumene hydroperoxide, *tert*-butyl hydroperoxide, and hydrogen peroxide. However, this enzyme, which does not conjugate CDNB with GSH (34), displays glutathione peroxidase activity toward cumene hydroperoxide, hydrogen peroxide, and lipid hydroperoxides and is distinct from the classical glutathione peroxidase (35). Evidence suggests that the enzyme is interfacial in character and can interact directly with liposomes to reduce phospholipid hydroperoxides (33). The addition of this protein to microsomal incubation mixtures inhibited lipid peroxidation (34). Substrate specificities indicate that this enzyme is distinct from the nuclear glutathione transferase (36). In addition to the selenium-dependent glutathione peroxidases, a related extracellular selenoprotein, selenoprotein-P, is found in plasma and in extracellular fluids (37). This monomeric 41 kD glycoprotein contains 10 selenocysteines, and enzyme levels are highly sensitive to changes in dietary selenium status. Although a specific antioxidant activity has not been established for this protein, it is postulated to exert antioxidant effects (37). The soluble intracellular form of Selenoprotein-P, selenoprotein-P₂, is generally regarded as an indispensable defense against hydrogen peroxide (31). Reduction of phospholipid hydroperoxides by phospholipid hydroperoxide glutathione peroxidase, together with peroxyl radical scavenging by vitamin E (discussed later), is thought to constitute a highly efficient defense against membrane lipid peroxidation (38). Weitzel and Wendel (39) have reported findings that phospholipid hydroperoxide-glutathione peroxidase activity regulates the activity of 5-lipoxygenase via regulating the tone of endogenous hydroperoxides. Nevertheless, the relative importance of these different selenoproteins in antioxidant protection remains poorly understood. Awad et al. (40) recently reported that severe dietary selenium deficiency reduced glutathione peroxidase activity to less than 2% of control values without increasing hepatic lipid peroxidation, whereas vitamin E deficiency did increase lipid peroxidation. In another experiment, diquat toxicity in chronically selenium deficient rats was reduced by pretreatment with selenium 12 h prior to diquat exposure. However, the selenium pretreatments did not affect either selenium glutathione peroxidase or phospholipid hydroperoxide glutathione peroxidase, but instead increased plasma levels of selenoprotein-P. A better understanding of the antioxidant role of selenium awaits a clarification of the true function of selenoprotein-P and extracellular selenium glutathione peroxidase and of their interplay with intracellular glutathione peroxidases.

Michiels et al. (41) have reviewed the importance of the defense enzymes S-glutathione peroxidase, catalase, and Cu,Zn-SOD for cell survival against oxidative

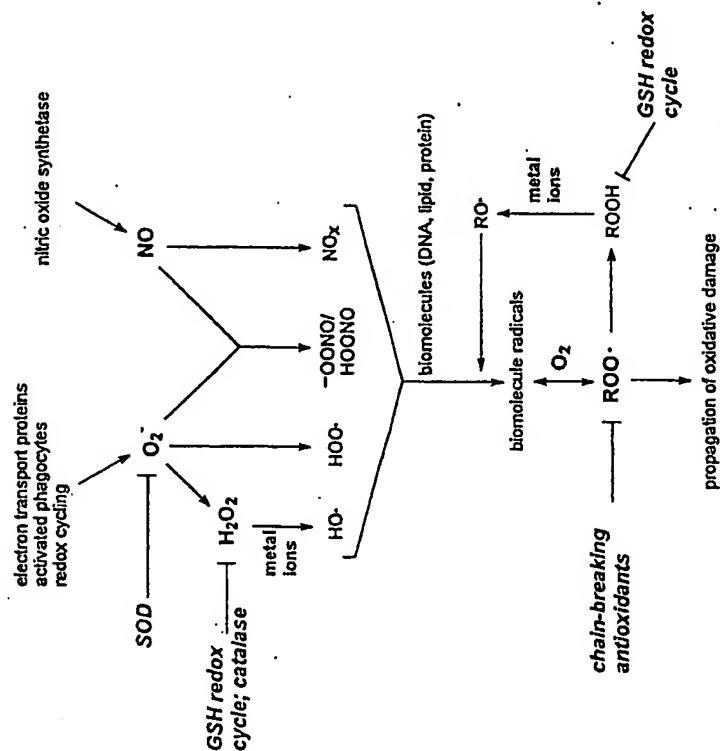


FIG. 1. Sites of blocking oxidant challenges by antioxidant defenses.

catalsis, since the nonenzymatic rate is approximately four orders of magnitude smaller at pH 7.4 (25). Mammalian SOD enzymes include a homodimeric Cu,ZnSOD in the cytosol and a homotetrameric MnSOD in mitochondria (26). Extracellular fluids contain a tetrameric, glycosylated form of the Cu,Zn enzyme (27). The mitochondrial MnSOD is highly inducible by cytokines such as tumor necrosis factor (28) and by mediators of acute oxidative stress, such as superoxide or hydrogen peroxide (29). Catalase is a hemoprotein found in peroxisomes of eukaryotic cells and catalyzes the conversion of hydrogen peroxide to water and oxygen. This enzyme also can be induced in response to cellular hydrogen peroxide exposure (29).

Glutathione peroxidases are selenoproteins found in essentially all tissues. Four isozymes of with glutathione peroxidase activity have been characterized: (a) the classical cellular glutathione peroxidase, GSHPx-1, (b) the phospholipid hydroperoxidase glutathione peroxidase, GSHPx-2, (c) the plasma glutathione peroxidase, GSHPx-3, and (d) GSHPx-GI (30).

The best known of these, glutathione peroxidase, is a homotetramer consisting of 22-kD subunits, each with one selenocysteine residue (31). The enzyme is found

stress. From the evidence, they suggest that each enzyme has a specific as well as an irreplaceable function. In part, roles of the enzymes were assessed with the aid of specific enzyme inhibitors including aminotriazole (42) for catalase, diethyldithiocarbamate (43) for Cu,Zn-SOD, mercaptosuccinate (44) for glutathione peroxidase, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (45) for glutathione reductase, and buthionine sulfoximine (BSO) (46) for GSH synthesis. In transfection studies in which overexpression of a specific enzyme is studied, the physiological responses indicate that the metabolism of reactive oxygen species may have a critical balance. For example, Mn-SOD-transfected mouse cells overexpressing SOD activity were found to be more resistant to hyperoxia (47) and paraquat (47,48). However, Cu,Zn-SOD-enriched bacteria displayed increased sensitivity to hyperoxia (49) and paraquat (50). Warner (51) concurs that SOD has an important role in defense against degenerative disease, but much remains to be understood concerning effects of manipulation of SOD expression as an intervention. Transfection experiments could therefore cause increased or decreased toxicity depending on relative concentrations of hydrogen peroxide and superoxide. In addition, iron (or copper) has the potential for a major influence by being optimized in its redox ratio ($\text{Fe}^{2+}/\text{Fe}^{3+}$) and involving various constituents for increasing the reactivity of hydrogen peroxide. Luo et al. (52) have proposed that hydrogen peroxide toxicity is associated with three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA.

Small-Molecule Antioxidants

Numerous small molecules (<1000 MW) with high reactivity toward oxidants have been described. Three of these, vitamin E, ascorbic acid, and glutathione, play essential antioxidant roles in tissues and in extracellular fluids. The functions of these are discussed in detail next. Numerous other molecules, including carotenoids (53,54), dihydrolipoic acid (55), flavonoids (56), plant polyphenols (57), and ubiquinol (58,59), exert antioxidant effects in vitro and may assume important antioxidant roles in vivo under conditions of increased dietary intake or pharmacological supplementation. Some products of intermediary metabolism, such as bilirubin (60) and uric acid (61), that are toxic at high levels may nevertheless exert antioxidant effects in some tissue microenvironments. Many drugs and other chemicals have been shown to have antioxidant properties in vitro and may also contribute to antioxidant defense under certain circumstances.

In contrast to antioxidant enzymes, which scavenge superoxide and hydrogen peroxide, small molecule antioxidants act for the most part as scavengers of secondary oxidants and inhibitors of radical chain propagation. Such molecules may be classified as *chain-breaking antioxidants*, which are defined as compounds that act by trapping peroxyl radicals. As discussed earlier, peroxyl radicals are the principal chain-propagating species encountered in oxidant challenges and probably the most common ultimate mediators of oxidative damage. Chain breaking antioxidants typi-

cally function in a two-step sequence in which the antioxidant traps one peroxyl radical to form an antioxidant-derived radical [Eq. (3)], which then traps a second radical to form nonradical products [Eq. (4)].



Each reaction terminates a radical chain and thus may prevent as many as 20 or more subsequent oxidations. Chain-breaking antioxidants can be effective at relatively low concentrations. For example, membranes typically contain about 1 α -tocopherol per 1000 phospholipids (62,63).

Because small-molecule antioxidants are known to react at high rates with many oxidants (hydroxyl radicals, peroxynitrite, alkoxyl radicals, peroxyl radicals), it perhaps seems surprising that these compounds should act primarily as chain-breaking antioxidants. The apparent specificity of most small-molecule antioxidants for reaction with peroxyl radicals as opposed to other radicals (e.g., hydroxyl radical) is due largely to kinetics. Peroxyl radicals react at relatively slow rates with many biomolecules (e.g., $k \sim 10^2 \text{ M}^{-1} \text{ s}^{-1}$ for linoleic acid (64), but at much higher rates with antioxidants (e.g., $k \sim 10^5\text{--}10^6 \text{ M}^{-1} \text{ s}^{-1}$ for α -tocopherol, depending on the reaction medium (65). Reaction of peroxyl radicals with the antioxidant is kinetically favored, even though the concentration of oxidizable lipid greatly exceeds that of the antioxidant. In contrast, hydroxyl radicals react with most biomolecules and antioxidants at near the diffusion controlled rate [$k \sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (66,67)], so there is no kinetic preference for reaction with the antioxidant. Indeed, since other biomolecules outnumber antioxidant molecules, quenching of hydroxyl radicals would occur infrequently. The antioxidant instead would be much more likely to trap peroxyl radicals formed subsequent to initial hydroxyl radical attack. In view of these considerations, the frequently used term "hydroxyl radical scavenger" is probably a misnomer, at least in reference to actions in vivo. Only when relatively high concentrations of scavenger are employed in vitro would effective scavenging of hydroxyl radicals occur.

Small molecule antioxidants react with radicals by a variety of mechanisms [Eqs. (5–10)]. Phenolic antioxidants such as α -tocopherol and thiols such as glutathione usually quench radicals by hydrogen atom transfer (although the reaction also may occur by rapid electron transfer followed by proton transfer) [Eqs. (5) and (6)] (23,68).



The water soluble antioxidants ascorbate and urate instead react by electron transfer [Eqs. (7) and (8)] (16,61).



Carotenoids react by a combination of electron transfer and radical addition to the carotenoid polyene system [Eqs. (9) and (10)] (69,70).

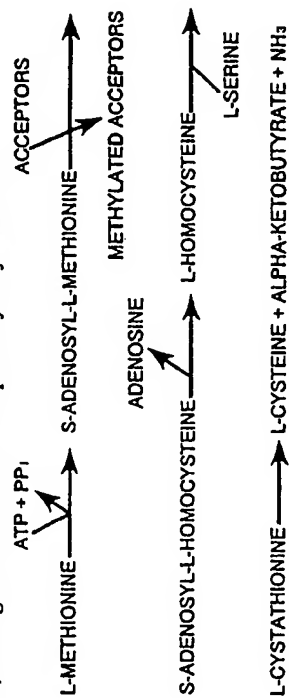


The radical intermediates generated by all these reactions may react with additional radicals in radical-radical termination reactions (e.g., reaction 4) or may undergo disproportionation or reductive "repair" reactions. These reactions of α -tocopherol, glutathione, and ascorbate and their radical intermediates are considered in detail in the following sections.

CELLULAR GLUTATHIONE AND OTHER THIOLS AS DEFENSE AND REPAIR AGENTS

Depending on the cell type, the intracellular concentration of glutathione is maintained in the range of 0.5–10 mM (71). Concentrations in the liver are 4–8 mM. Because of the redox status of glutathione that is maintained by intracellular glutathione reductase and NADPH, nearly all the glutathione is present as reduced glutathione (GSH) with less than 5% of the total is present as glutathione disulfide (GSSG). Constant production of GSSG is a result of continual endogenous production of superoxide from oxygen leading to the formation of hydrogen peroxide and lipid hydroperoxides. The GSH content of various organs and tissues represents at least 90% of the total nonprotein, low-molecular-weight thiols. The GSH content of liver is nearly twice that found in kidney and testes and over threefold greater than in the lung. The importance of hepatic GSH for protection against free radicals has been reviewed extensively (72–75).

The cystathionine pathway is of major importance to pathways of free-radical generation that can cause loss of GSH. Depletion of GSH by rapid conjugation can increase synthesis of GSH to rates as high as 2–3 $\mu\text{mol/h/g}$ wet liver tissue (76). The cysteine pool in the liver, which is about 0.2 $\mu\text{mol/g}$, has an estimated half-life of 2–3 min at such high rates of synthesis of GSH (72). Although the cystathionine pathway appears to be highly responsive to the need for cysteine biosynthesis in the liver, the organ distribution of the pathway may be limited.



In mammals, such as rats, the liver is the main site of cysteine biosynthesis, which occurs via the cystathionine pathway as shown in the preceding scheme. Maintenance of high concentrations of GSH in the liver, in association with high rates of GSH secretion into plasma and extensive extracellular degradation of GSH and GSSG, supports the concept that liver GSH is a physiological reservoir of cysteine. This idea, which was proposed originally by Tateishi et al. (77) and Higashi et al. (78), was that cells possess two pools of GSH. One has a fast (2-h) and the other a slow (30-h) turnover (78,79). Meredith and Reed (80) observed that in freshly isolated rat hepatocytes the mitochondrial pool of GSH (about 10% of the total cellular pool) had a half-life of 30 h while the half-life of the cytoplasmic pool was 2 h. They concluded that the mitochondrial pool might represent the stable pool of GSH observed in whole animals. Further, cysteine has a sparing effect on the requirement of the essential amino acid methionine in the rat (81). This observation is in agreement with the unidirectional process of trans-sulfuration in which methionine sulfur and serine carbon are utilized in cysteine biosynthesis via the cystathionine pathway. For reviews see Reed and Beatty (72) and Reed (75)].

In vivo treatment of rats with an inhibitor of γ -glutamyl transpeptidase (AT-125) prevents degradation of GSH in plasma leading to massive urinary excretion of GSH (82a). This treatment also lowers the hepatic content of GSH because it inhibits recycling of cysteine to the liver (80). A physiologic decrease in interorgan recycling of cysteine to the liver for synthesis of GSH also may account in part for the decrease of hepatic GSH during starvation and for the marked diurnal variation in concentration of GSH in liver. The nadir occurs in the late afternoon, whereas the early morning peak occurs shortly after the animals are fed. The efflux of liver GSH and metabolism of the resulting plasma GSH and GSSG appears to help insure a continuous supply of plasma cysteine. This cysteine pool should in turn minimize the degree of fluctuation of GSH concentrations within the various body organs and cell types that require only cysteine or cystine, or both, rather than methionine for synthesis of GSH.

Glutathione deficiency can be achieved in vivo by the administration of BSO. When newborn rats or guinea pigs are treated with BSO a GSH deficiency develops and the animals develop multiorgan failure and die within a few days. Death can be prevented by the administration of ascorbate (82b).

A controversial approach to assessing the potential for chemicals to cause free-radical damage in vivo is to chemically intoxicate an intact animal and then to measure products of lipid peroxidation in microsomes prepared from the intoxicated animal. In this manner, the depletion of glutathione in vivo with agents that form glutathione conjugates enhances subsequent lipid peroxidation in vitro. Results from such experiments show consistently that an in vivo threshold of 1 $\mu\text{mol GSH/g}$ liver is associated with spontaneous lipid peroxidation in microsomes (83). This critical value of GSH is about 20% of the initial concentration of GSH. Addition of exogenous GSH inhibited the lipid peroxidation in vitro in a concentration-dependent manner; 1 mM GSH yielded 50% inhibition. There also is observed a strong enhancement of spontaneous lipid peroxidation in phenobarbital-induced rats.

The role of GSH as a defense against oxidative stress generated by free radicals has been quantified by the measurement of lipofuscin. A hypothesis for lipofuscinogenesis that was postulated by Brunk et al. (84) involves the interplay of two processes: (1) the intracellular production of superoxide and hydrogen peroxide and (2) secondary lysosomes that degrade lipids and proteins to a poorly defined substance known as lipofuscin (85). The loss of GSH by BSO treatment of cardiac myocytes resulted in an increase in lipofuscin that appears associated with loss of GSH-dependent defense against increased levels of hydrogen peroxide (85).

GLUTATHIONE REDOX CYCLE DEFENSE AGAINST FREE-RADICAL-INDUCED EVENTS

A major defense system against endogenous reactive oxygen species is the glutathione redox cycle (Figure 2) (for a review see Reed, 86). Support for the role of the glutathione redox cycle in defense is that GSH depletion to about 20–30% of normal level of glutathione can impair the cell's defense against the toxic actions of both biological reactive intermediates and reactive oxygen species and may lead to cell injury and death. Endogenous free-radical production, which is a normal physiological process, is a consequence of aerobic metabolism that occurs mostly in the mitochondria of eukaryote cells. Mitochondrially generated H_2O_2 , if not decomposed, can lead to the formation of radicals that cause damage to membranes, nucleic acids, and proteins, and alter their functions. A major protective role against exogenous free radicals, which are generated by bioreduction of many xenobiotics followed by redox cycling, is also provided by the ubiquitous glutathione redox cycle (Figure 2). This cycle utilizes NADPH- and, indirectly, NADH-reducing equivalents in the mitochondrial matrix as well as the cytoplasm to provide GSH by the glutathione reductase-catalyzed reduction of GSSG. When the glutathione redox cycle is functioning at maximum capacity to eliminate hydrogen peroxide, a major regulatory effect is imposed on other NADPH-dependent pathways.

Glutathione reductase, which is important in the defense against oxygen-derived free radicals by GSH, is itself regulated by the redox status of the cell. Being similar to other reductases such as nitrate, nitrite, and $NADP^+$ reductase, GSH reductase is inactivated upon reduction by its own electron donor, NADPH. It has been proposed that this autoinactivation of glutathione reductase by NADPH and the protection as well as reactivation by GSSG regulates the enzyme *in vivo* (87). The activity of glutathione reductase may reflect the physiological needs of the cell especially during oxidative stress. For example, 40–50 μM intracellular NADPH inactivates glutathione reductase in the absence of GSSG and decreases glucose metabolism via the hexose monophosphate pathway. The physiological ratio of GSSG:GSH should provide sufficient GSSG at this level of NADPH to permit retention of significant glutathione reductase activity by preventing inactivation (87).

Cytosolic glutathione peroxidase is a selenium-dependent enzyme that is extremely specific for glutathione and is capable of rapidly detoxifying hydrogen

CELLULAR PROTECTIVE SYSTEMS

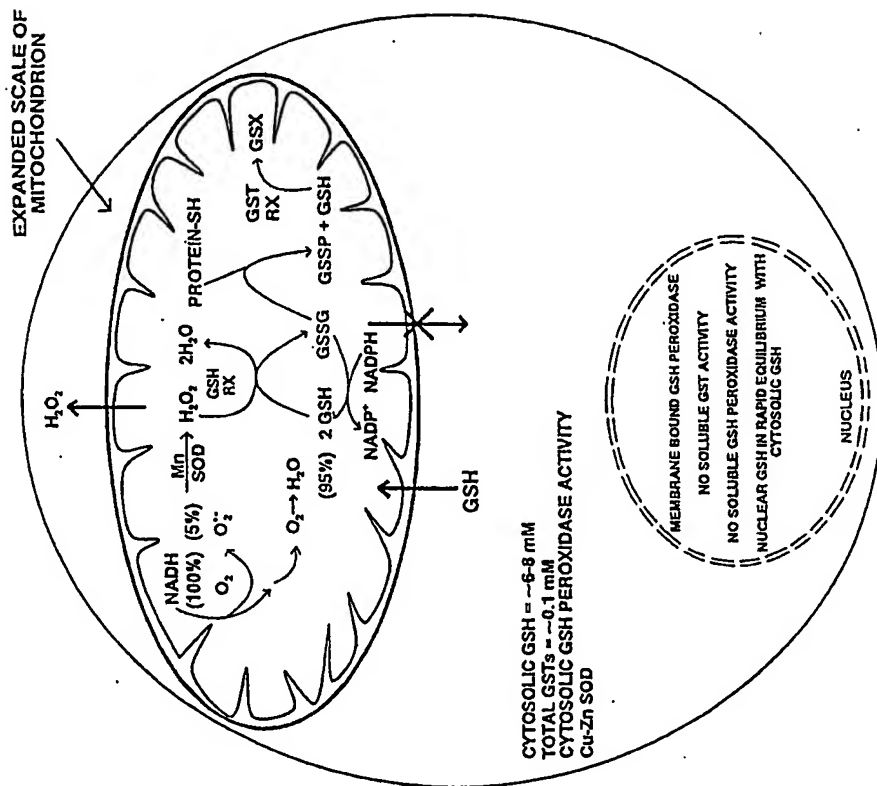


FIG. 2. Cellular protective systems and the glutathione redox cycle. MnSOD in the mitochondria and Cu-ZnSOD in the cytosol provide enzymatic conversion of superoxide to hydrogen peroxide, which is detoxified by the glutathione redox cycle components glutathione reductase and peroxidase present in the mitochondria matrix (shown) and the cytosol (not shown).

peroxide and certain hydroperoxides as a partner in the glutathione redox cycle with glutathione reductase. As mentioned earlier, selenium-dependent glutathione peroxidase activity is the result of the expression of multiple isozymes.

The antitumor benzantraquinone, adriamycin, undergoes rapid bioreduction by NADPH-dependent cytochrome P-450 reductase with concomitant consumption of

oxygen (88). Adriamycin cytotoxicity may be the result of free radicals formed by bioreduction that overwhelm the cellular antioxidant capacity, including that portion provided by the glutathione redox cycle. Inactivation of glutathione reductase with BCNU has permitted the demonstration of the protective role of the glutathione redox cycle against an adriamycin-mediated challenge (89,90). Depletion of GSH concurrently with inactivation of glutathione reductase can enhance the cellular injury mediated by adriamycin-generated radicals in isolated hepatocytes (90).

The intracellular concentration of GSH in isolated hepatocytes has been examined under conditions that result in enhanced free-radical production. Production of malondialdehyde, which is an index of lipid peroxidation, can be stimulated by addition of a glutathione depletor, diethyl maleate (89). This observation suggests that intracellular concentrations of GSH under these conditions are important for membrane and cellular integrity. That is, GSH protects against free-radical damage to unsaturated fatty acid moieties in biological membranes.

Defense by Glutathione S-Transferases

GSH-dependent protection against lipid peroxidation has been demonstrated in mitochondria (91-93), nuclei (94), microsomes (91,93-98), and cytosol of rat liver (99-101). Lipid peroxidation induced in mitochondria also is inhibited by respiratory substrates such as succinate, which leads indirectly to reduction of ubiquinone to ubiquinol. The latter is a potent antioxidant (102-104). The essential factor in preventing accumulation of lipid peroxides and lysis of membranes in mitochondria, however, is glutathione peroxidase (105). Although the prevention of free-radical attack on membrane lipids may occur by an electron shuttle that utilizes vitamin E and GSH in microsomes, similar activity may not be capable of inhibiting peroxidation in mitochondria (96,106). Instead, mitochondrial GSH S-transferase(s) may prevent lipid peroxidation in mitochondria by a non-selenium glutathione-dependent peroxidase activity. Three GSH S-transferases have been isolated from the mitochondrial matrix (107), and nearly 5% of the mitochondrial outer membrane protein consists of microsomal glutathione S-transferase (108). GSH S-transferase in the outer mitochondrial membrane could provide the GSH-dependent protection of mitochondria by scavenging lipid radicals by a mechanism that requires vitamin E and is abolished by bromosulphthalein (108).

A limited number of studies have focused on the susceptibility of the cell nucleus to lipid peroxidation. The nuclear membrane regulates the transport of mRNA into the cytoplasm and aids in the process of nuclear division. DNA is also frequently associated with certain regions of the nuclear membrane (109), and it seems likely that nuclear membrane peroxidation may disrupt many of these critical functions. The proximity of the nuclear membrane to DNA could also contribute to the interaction of DNA with reactive compounds generated in lipid peroxidation. Several studies indicate that such lipid peroxidation products can alter the structure and function of DNA (110-113). This fact is of importance since hydroxyl radicals diffuse an average of only 60 Å before reacting with cellular components (114). Assays for

8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage include *in vivo* studies with urine samples (115). Nuclear peroxidation may also increase interactions between more stable peroxidation products and DNA. The cytosolic enzymes aldehyde dehydrogenase (116), glutathione transferase (117), and glutathione peroxidase (91) have all been shown to metabolize various reactive lipid peroxidation products. Such cytosolic enzymes may metabolize peroxidation products generated throughout the cell before they diffuse into the nucleus and interact with DNA.

Glutathione protection of isolated rat liver nuclei against lipid peroxidation is abolished by exposing isolated nuclei to the glutathione transferase inhibitor S-octylglutathione (36). S-Octylglutathione also inhibited nuclear glutathione transferase activity and glutathione peroxidase activity. A large percentage of the glutathione transferase activity associated with isolated nuclei was solubilized with 0.3% Triton X-100. Since this treatment removes nuclear membranes while preserving the integrity of the remaining nucleus, it appears that the peroxidase activity is associated with the nuclear membrane. This activity in conjunction with GSH may contribute to the inhibition of lipid peroxidation in nuclear membranes and thereby preserve the integrity of this important membrane system. Increasing evidence suggests that this inhibition of peroxidation may in turn protect the structure and function of DNA.

Endogenous α -tocopherol levels in isolated rat liver nuclei have been measured and found to be 0.045 mol E (mol α -tocopherol per mol phospholipid \times 100) (36). This value corresponds to 970 polyunsaturated fatty acid (PUFA) moieties to one molecule of α -tocopherol in the nuclear membrane. These values are higher than values reported for rat liver microsomes (3313) (63) and mitochondria (2100) (118). A threshold level of 0.085 mol% for the prevention of NADPH-induced lipid peroxidation was established for isolated nuclei. That value could be lowered to 0.040 mol% when 1 mM GSH was added to assist in the inhibition of lipid peroxidation. The ability of GSH to enhance α -tocopherol-dependent protection against nuclear lipid peroxidation appears to be mainly by a "sparing" effect on the near threshold level of α -tocopherol in the nuclear membrane.

Since lipid hydroperoxides can initiate lipid peroxidation, the reduction of these compounds can contribute to the inhibition of peroxidation (Figure 1). If glutathione peroxidase activity is associated with the phospholipid bilayer of the nuclear membrane, such an association may contribute to the ability of the peroxidase to reduce lipid hydroperoxides. Thus, the association of a glutathione-dependent peroxidase with membranes may encourage the reduction of lipid hydroperoxides located within lipid bilayers.

Glutathione Compartmentation and Defense Against Free-Radical-Induced Injury: Mitochondrial GSH

Several studies have shown that mitochondrial GSH functions as a discrete pool separate from cytosolic GSH. A report by Jocelyn (119) demonstrated that

mitochondrial GSH is impermeable to the inner membrane following isolation of mitochondria. However, Kurosawa et al. (120) have reported the transport of glutathione across mitochondrial membranes. Garcia-Ruiz et al. (121) have evidence that the rat hepatic mitochondrial carrier for reduced glutathione (GSH) transport is distinct from the sinusoidal and canalicular transporters. Wahländer et al. (122) reported the concentration of mitochondrial GSH (10 mM) is higher than cytosolic GSH (7 mM). As previously mentioned, studies by Meredith and Reed (80) demonstrated different rates of GSH turnover in the cytosol and mitochondria confirming the existence of separate intracellular GSH pools. The ratio of GSH:GSSG in mitochondria is approximately 10:1 under normal (untreated) conditions. As reported by Olafsdottir et al. (123), unlike cytosolic GSSG, GSSG is not effluxed from the mitochondrial matrix compartment. This study demonstrated that during oxidative stress induced with *t*-butyl hydroperoxide, GSSG is accumulated in the mitochondrial matrix and eventually reduced back to GSH. However, as the redox state of the mitochondria increases, an increase in protein mixed disulfides is also observed. This study concluded that mitochondria are more sensitive to redox changes in GSH:GSSG than the cytosol and therefore mitochondria may be more susceptible to the damaging effects of oxidative stress. These findings suggest that under certain experimental conditions, irreversible cell injury due to oxidative challenge may result from irreversible changes in mitochondrial function.

Mitochondria play a critical role in cellular defense free radicals and associated nonradical oxidants. One of the difficulties in experimentally demonstrating the essential role of mitochondrial GSH has been the inability to selectively deplete the mitochondrial pool of GSH. Recently, Shan et al. (124) have utilized the mitochondrial 3-hydroxybutanoate dehydrogenase to generate a GSH-depleting agent, 3-oxo-4-pentenoate, from (R,S)-3-hydroxy-4-pentenoate. By a Michael acceptor reaction of 3-oxo-4-pentenoate with GSH, the mitochondrial GSH pool is selectively depleted. Because mitochondria are a major site for the generation of reactive oxygen species they are susceptible to injury from free radicals. Because of the lack catalase in mitochondria, the entire burden for defense is dependent upon the glutathione redox cycle. Shan et al. (124) observed that the depletion of the mitochondrial, but not the cytosolic, glutathione pool potentiated the cytotoxicity of *tert*-butyl hydroperoxide. As first proposed by Meredith and Reed (80,90), oxidant and electrophile cytotoxicity was correlated with the depletion of GSH in mitochondria but not the cytosol. For reviews see Reed (73-75). In agreement with the mitochondria being a target for free-radical injury, transgenic mice that express high levels of Mn-SOD activity in mitochondria are protected from adriamycin-induced cardiac toxicity (125).

Since mitochondria contain the enzymes and cofactors necessary for the GSH/GSSG redox cycle (126) but do not contain catalase (127), we may assume that a primary function of mitochondrial glutathione (GSH) is the detoxification of endogenously produced H_2O_2 . This redox cycle also protects protein sulfhydryls from oxidation (128).

The mitochondrial glutathione redox cycle has a role in regulating mitochondrial

oxidations. Various oxidants decrease O_2 uptake by isolated mitochondria and cause a complete turnover of GSH via glutathione peroxidase every 10 min (129). It appears that a continuous flow of reducing equivalents through the glutathione redox cycle is balanced by a continuous formation of mitochondrial NADPH, which is needed for glutathione reductase activity. In addition, metabolism of hydrogen peroxide in mitochondria poses a regulatory function in regard to the oxidation of substrates by lipamide-dependent ketoacid oxidases (129), which generate NADPH-reducing equivalents. The entire NADPH:NADP⁺ pool may turn over at least once every minute during a maximum oxidant challenge.

The extent to which bioreduction utilizes mitochondrial reducing equivalents is still uncertain, but evidence is increasing that such effects are very important and relate to both calcium and protein thiol homeostasis of mitochondria. For example, the loss of NADPH, which occurs following addition of menadione to isolated mitochondria, is not related to its consumption via the glutathione redox cycle but more likely is related to bioreductive metabolism by NADH-ubiquinone oxidoreductase (130,131).

Protein Thiols and Toxicity

Thiol groups are well known to be important for normal protein functions, and increasing evidence supports the vital importance of these thiols for cell viability during cytotoxic events. Protein inactivation by oxidation of protein cysteinyl thiols has been shown to occur in more than 240 enzymes (132,133). Membrane-bound enzymes are damaged during lipid peroxidation, and evidence of vitamin E protection strongly supports a free-radical mechanism for protein damage via oxidative stress (134). Oxidative stress can cause loss of protein functions by damaging amino acid residues other than cysteine including methionine, tryptophan, and histidine. An important aspect of such damage is that lipid peroxidative events can amplify free-radical processes that propagate chain reactions. Failure to terminate free-radical processes with a chain-breaking antioxidant, such as vitamin E, can lead to 4 to 10 propagation events occurring per initiation and thus each initiation is amplified (92). Since the reduction of lipid hydroperoxides by GSH utilizes NADPH for the regeneration of GSH from GSSG, the rate of NADPH production can be limiting during oxidative stress (86). Therefore GSSG may be transported from the cell, especially the liver parenchymal cell, when not reduced due to limited levels of NADPH (135). Decreased availability of NADPH and GSH can impair other GSH-dependent detoxication pathways including metabolism of hydrogen peroxide (6), decreased protection of thiols in protein (136,137), and decreased reaction with free radicals (97). Thus, energy-dependent processes involving NADPH, GSH, and thiols in proteins appear to be critically involved in cellular homeostasis during chemical-induced toxicity.

ANTIOXIDANT PROTECTION BY VITAMINS E AND C

Vitamin E is the name given to a family of natural products comprising the tocopherols and tocotrienols. The most potent of these in animal bioassays is α -tocopherol, which has the highest rate constant for reaction with peroxyl radicals (138). Tocopherols are not synthesized *de novo* in animals, and tissue levels generally reflect dietary intake of grains and plant-derived oils, which are the best natural sources. The liver plays a central role in α -tocopherol distribution by incorporating dietary α -tocopherol into lipoproteins, which then deliver α -tocopherol to tissues. Other tocopherols, principally γ -tocopherol, may be delivered directly to tissues via chylomicrons.

Dietary α -tocopherol deficiency enhances the susceptibility of biological membranes to oxidative damage *in vitro* and *in vivo* (for a review, see Chow, 139). Diplock (140), and Rice-Evans and Diplock (141) have reviewed the status of antioxidant nutrients and disease prevention. This section focuses on the interaction of antioxidants for protection against free-radical damage.

α -Tocopherol Threshold and Antioxidant Effect

Although regulation of α -tocopherol distribution at the cellular level is poorly understood, membrane α -tocopherol levels typically range from one to four α -tocopherol molecules per 1000 phospholipids (62,63). This range is thought to correspond to an antioxidant threshold for α -tocopherol (142). Several *in vitro* studies have demonstrated that α -tocopherol provides effective antioxidant protection only at levels above a threshold concentration (reviewed by Liebler, 143). The α -tocopherol threshold derives from (1) the tendency of α -tocopherol to inhibit peroxyl radical propagation and (2) the tendency of peroxyl radicals to consume α -tocopherol. The threshold essentially represents the α -tocopherol level at which these two opposing tendencies are in balance. Because membrane α -tocopherol levels are apparently kept close to a threshold concentration, relatively modest α -tocopherol depletion could compromise membrane antioxidant defense. Under some circumstances, regeneration of α -tocopherol from its oxidation products may prevent critical depletion of membrane α -tocopherol (discussed later). Moreover, *in vitro* studies in a liposome model demonstrated that antioxidant synergism between α -tocopherol and ascorbate was most efficient when membrane α -tocopherol levels exceeded the threshold concentration experimentally determined for that system (142). An apparent α -tocopherol threshold for protection against lipid peroxidation *in vivo* has been deduced from plasma α -tocopherol levels in rats (144,145).

Antioxidant Reactions

TH exerts antioxidant effects primarily by trapping peroxyl radicals [Eqs. (11) and (12)]. α -Tocopherol reacts readily with peroxyl radicals to yield a hydroperoxide and the resonance-stabilized tocopheroxyl radical (T).

FREE-RADICAL DEFENSE AND REPAIR



The tocopheroxyl radical formed in reaction 11 may be reduced by several biochemical reductants and this is postulated to regenerate α -tocopherol and complete a one-electron redox cycle (discussed later). Direct observation of reactions 11 and 12 in biological systems generally is not feasible, but much has been learned about α -tocopherol antioxidant reactions through analyses of the products formed in reaction 12.

Further reactions of the tocopheroxyl radical yield two groups of nonradical products (146-151). The first consists of 8 α -substituted tocopherones, which result either from peroxyl radical addition at C-8 α to form 8 α -(alkyldioxy)tocopherones (1, Figure 3) (146,148,149,152) or from electron transfer followed by hydrolysis to yield 8 α -(hydroxy)tocopherones 2 (151,153,154). Products 1 and 2 hydrolyze and rearrange to form α -tocopherolquinone 3 (155). Formation of 8 α -substituted tocopherones 1/2 is analogous to reactions of simple antioxidant phenols (e.g., BHT) with peroxyl radicals and is compatible with the previously reported stoichiometry of two peroxyl radicals trapped for each α -tocopherol oxidized (138,146,156).

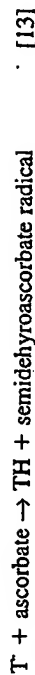
The remaining products consist of epoxytocopherones 4/5 and their hydrolysis products 5,6-epoxy- α -tocopherolquinone 6 and 2,3-epoxy- α -tocopherolquinone 7, respectively (149,157). Although the mechanism of α -tocopherol oxidation to epoxides 4/5 is not known, recent studies of product yield and antioxidant stoichiometry indicated that epoxide product yields vary considerably with reaction environment, but the antioxidant stoichiometry remains essentially unchanged at two radicals scavenged per α -tocopherol consumed (158,159).

Peroxyl radical scavenging by α -tocopherol thus forms 8 α -substituted tocopherones and epoxytocopherones, which then hydrolyze to more stable α -tocopherolquinone and epoxyquinone products. In recent studies of the peroxyl radical mediated oxidation of α -tocopherol in microsomes *in vitro*, tocopherone intermediates were found to account for over half of the α -tocopherol consumed (160). Mild acid treatment of microsomal incubation samples effected complete conversion of the tocopherone precursors to α -tocopherolquinone and epoxyquinones (151), which were analyzed by a sensitive stable isotope dilution GC-MS method (160). Analysis of α -tocopherol, together with α -tocopherolquinone, its reduction product α -tocopherolhydroquinone (discussed later), and the epoxyquinones can provide a "snapshot" of the redox distribution of α -tocopherol and its major oxidation products.

Redox Cycles for Vitamin E: One-Electron Redox Cycle

Early observations by Golumbic and Mattili (161) and by Tappel et al. (162) led to the suggestion that α -tocopherol may be regenerated from its oxidation intermediates by other biochemical reductants and that this redox chemistry would maintain through ongoing oxidative stress. The most widely considered redox cycle

is the one-electron redox cycle in which α -tocopherol is oxidized by a radical to the tocopheroxyl radical (eq 1), which is then reduced back to α -tocopherol by a reductant such as ascorbate:



Proof of the chemical feasibility of reaction 13 came from pulse radiolysis work by Packer, Slater, and Willson (163). However, evaluation of this one-electron redox cycle in biological systems has been much more difficult. The large body of work on this problem has been reviewed in detail from different perspectives (143,164-166). Several general observations are presented here.

First, in the numerous reported demonstrations of α -tocopherol "sparing" by ascorbate, other low-molecular-weight antioxidants, and redox proteins, the extent to which these co-antioxidants acted by regenerating α -tocopherol or by directly trapping radicals generally was not assessed. This makes it difficult to attribute protection against α -tocopherol depletion to tocopheroxyl radical recycling *per se* or to independent antioxidant actions of the co-antioxidants. Indeed, it seems likely that both tocopheroxyl radical recycling and direct, co-antioxidant effects of ascorbate may occur to varying degrees in different environments. It is nevertheless noteworthy that in a carefully conducted study of α -tocopherol turnover in several tissues of the guinea pig, dietary ascorbate status did not measurably affect the kinetics of α -tocopherol turnover (167).

Second, reduction of the tocopheroxyl radical by ubiquinol, ascorbate, or other co-antioxidants has been unambiguously demonstrated in human low-density lipoproteins (59). These co-antioxidants reverse the novel pro-oxidant effect of the tocopheroxyl radical in the lipoprotein particle by reducing the tocopheroxyl radical to α -tocopherol and thus "carrying away" the radical from the lipoprotein. In the absence of a coreductant, the tocopheroxyl radical may actually initiate peroxidation of lipoprotein lipid (168).

Third, glutathione and other low-molecular-weight thiols apparently do not reduce the tocopheroxyl radical directly, but instead act to regenerate other antioxidants, such as ascorbate, which then may reduce the tocopheroxyl radical (166,169). Enzymatically mediated synergism between glutathione and α -tocopherol most likely results from parallel antioxidant actions of glutathione peroxidase enzymes (selenium-dependent glutathione peroxidase, selenium-dependent phospholipid hydroperoxide glutathione peroxidase, and some glutathione *S*-transferases) (38).

Redox Cycles for Vitamin E: Two-Electron Cycle

Tocopheroxyl radicals that do not undergo a one-electron reduction to α -tocopherol may instead either disproportionate or react with peroxyl radicals to form a variety of products, as discussed earlier. A large fraction of the products formed are 8a-substituted tocopherones, which may undergo reduction to α -tocopherol to complete a two-electron redox cycle (151,153-155). The possibility that a two-electron redox

cycle may contribute to α -tocopherol function has been raised previously and considered in detail recently (143). Although 8a-substituted tocopherones are easily reduced to α -tocopherol *in vitro* by ascorbate or nordehydroguaiaretic acid at acidic pH, it is not clear whether an enzyme-catalyzed reduction also can take place in biological systems. Of interest in this regard are the findings of Chan and colleagues, who described the oxidation of α -tocopherol in platelets to a product that was converted back to α -tocopherol by subsequent addition of ascorbate, glutathione, or nordehydroguaiaretic acid (170). Although the authors proposed that the reducible intermediate was the tocopheroxyl radical, the conditions of the experiment make it more likely that the intermediates were 8a-substituted tocopherones instead, as we have suggested previously (151). This suggests that a two-electron redox cycle involving 8a-substituted tocopherones could contribute to α -tocopherol maintenance during oxidative stress.

α -Tocopherol Esters

Esterified forms of α -tocopherol are resistant to oxidation and display improved stability over α -tocopherol. These forms of vitamin E, particularly α -tocopherol acetate, are frequently used in formulating vitamin supplements and other vitamin E-supplemented products. The esters themselves are inactive as antioxidants. When taken orally, α -tocopherol esters are efficiently hydrolyzed to the active antioxidant α -tocopherol by esterases in the gut, and bioavailability as free α -tocopherol is identical to an equal amount of the unesterified vitamin (171,172). In dosage by other routes, the ester and free α -tocopherol may not be bioequivalent. For example, topical application of free α -tocopherol inhibits photocarcinogenesis induced by repeated exposures to UV-B in a mouse model, whereas topical α -tocopherol acetate is ineffective (173). Polar α -tocopherol esters (e.g., α -tocopherol succinate) have been used in a number of studies *in vitro* to achieve α -tocopherol supplementation in cell culture systems (136,137,174,175). The water-dispersible hemisuccinate ester confers greater protection against oxidative stress than does either free α -tocopherol or α -tocopherol acetate (176,177). This apparently reflects a greater ability of the water-dispersible ester to release α -tocopherol in proximity to vital locations within cells (178).

Redox Chemistry of α -Tocopherolquinone

α -Tocopherolquinone (8, Figure 3), a stable end-product of α -tocopherol oxidation (discussed earlier) undergoes facile two-electron reduction by ascorbate, sodium borohydride, or other reductants to α -tocopherolhydroquinone at neutral pH (179,180). This hydroquinone would be expected to exert antioxidant effects similar to those of ubiquinols, which have been shown to be effective chain-breaking antioxidants. Hayashi et al. (181) demonstrated that isolated rat hepatocytes contained both α -tocopherolquinone and α -tocopherolhydroquinone in comparable

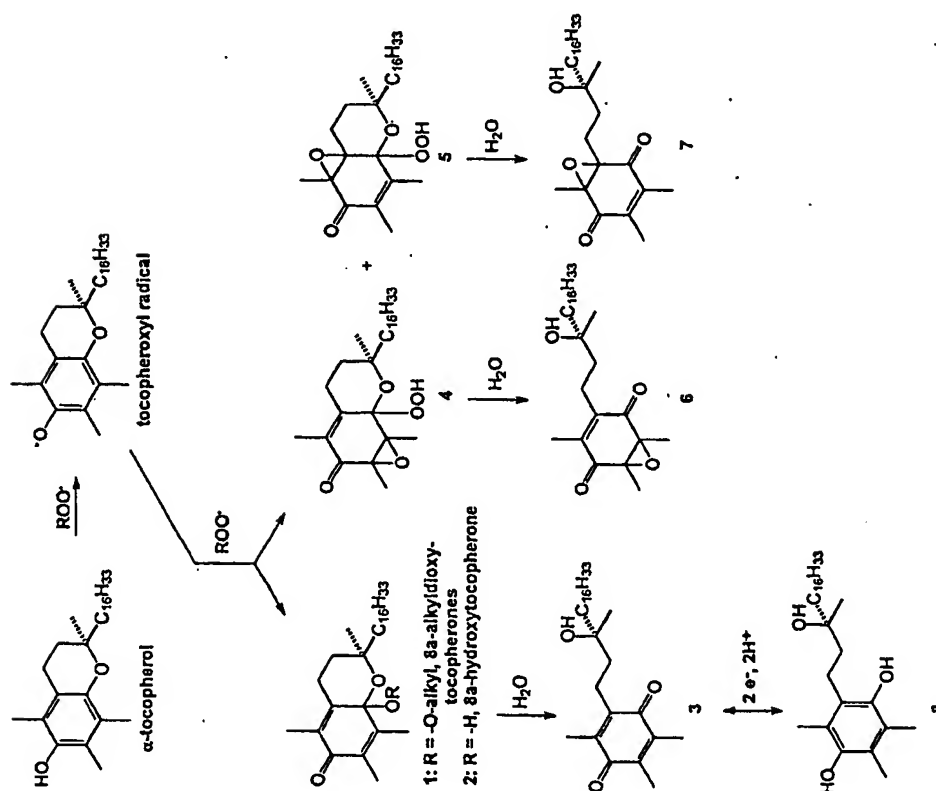


FIG. 3. Reaction products formed during the trapping of peroxyl radicals with α -tocopherol.

amounts and that the cells were capable of rapidly reducing exogenously added α -tocopherolquinone to the hydroquinone. Relatively little is known about the fate and possible role of α -tocopherolquinone formed by oxidative α -tocopherol turnover. Reduction of the quinone to the hydroquinone could provide an important contribution to cellular antioxidant protection, as proposed recently by Kohar et al. (182).

Antioxidant Protection by Vitamin C

In addition to its probable participation in the recycling of α -tocopherol (discussed earlier), ascorbate is thought to exert direct antioxidant effects. This follows from

the high reactivity of ascorbate as a one-electron reductant for many biologically relevant oxidants (16). The ability of ascorbate to exert both pro-oxidant and antioxidant effects complicates interpretations of its role as a cellular protectant. Antioxidant effects may be due either to (1) direct reaction with free radicals or nonradical oxidants to produce less reactive products (see Frei, 183, for a recent review), (2) regeneration of phenolic antioxidants such as α -tocopherol (discussed earlier), or (3) shifting the redox balance of transition metal redox couples to disfavor their participation in pro-oxidant reactions (184). As discussed earlier, some combination of the first two mechanisms probably accounts for antioxidant synergy between ascorbate and α -tocopherol. In most experimental systems, it may not be possible to distinguish the relative contributions of these mechanisms. Pro-oxidant effects of ascorbate are often observed in *in vitro* systems and are thought to be due to (1) reduction of transition metal ions to facilitate participation in Fenton chemistry (see Koppenol, chapter 1, this volume) and (2) formation of reactive oxygen species subsequent to metal catalyzed ascorbate autooxidation. It seems clear that ascorbate can inhibit or enhance metal catalyzed oxidations, depending on the ascorbate concentration (184).

The balance between pro-oxidant and antioxidant effects of ascorbate also may be controlled by the membrane status of α -tocopherol. In studies with a liposome model, in which oxidation was initiated by Fe^{2+} - and hydrogen peroxide, ascorbate alone at concentrations of less than 1 mM exerted a marked pro-oxidant effect, apparently by contributing to Fenton chemistry (142). Inclusion of α -tocopherol at a concentration of 0.2 mol%, which is above the α -tocopherol antioxidant threshold for that system, reversed the pro-oxidant effect of ascorbate. This coincided with an ascorbate-dependent prevention of α -tocopherol depletion. In liposomes containing α -tocopherol at lower concentrations, ascorbate was unable either to prevent α -tocopherol depletion or to prevent lipid peroxidation.

Intracellular ascorbate is consumed and recycled by permeant oxidants in activated neutrophils (185). Since free-radical generation by neutrophils can be as high as 200 nmole/ 10^6 cells/h (10), recycling of ascorbate occurs with rapid reduction of dehydroascorbate by a yet unknown mechanism for reduction. The accumulation of ascorbate and dehydroascorbate occurs by separate mechanisms (11). Winkler et al. (186) recently reviewed the literature on mechanisms for ascorbate reduction in cells. They concluded that there is little convincing evidence for the existence of putative NADH-dependent semidehydroascorbate reductases in mammalian cells, despite the fact that these enzymes are well described in plants. These authors concluded that semidehydroascorbate formed by one-electron oxidations of ascorbate disproportionates to dehydroascorbic acid, which then either is reduced nonenzymatically by glutathione or hydrolyzes to nonreducible products. Other mechanisms may maintain reduced ascorbate, including protein disulfide isomerase and glutaredoxin, which have dehydroascorbate-reducing activity (187) that is dependent on reduced glutathione. Welch et al. (11) have shown that in neutrophils dehydroascorbate reduction is protein mediated and chemical reduction by GSH could not account for the reduction. Also, they have found that dehydroascorbate transport and accumulation is 10-fold greater than for ascorbate. These findings support the concept of

extracellular defense against free radicals and other oxidants being an important aspect of the utilization and recycling of ascorbate, with intracellular reducing equivalents being made readily available by a glutathione-dependent reduction process (188).

CONCLUSIONS

Over the past two decades, a large body of work has helped to explain the functions of biological antioxidants and antioxidant enzyme systems. What has more recently emerged is a broader picture of the integration of biological antioxidant defense. Just as biological oxidant challenges encompass a diverse array of primordial oxidants, secondary oxidants, and propagating radicals, so do biological antioxidants comprise a multitiered defense. Specialization in antioxidant function allows specific enzymes and small molecules to scavenge specific oxidants with high efficiency. A diverse antioxidant defense system permits cells to defend against multiple components of oxidant challenges.

In a review of antioxidant therapy, Rice-Evans and Diplock (141) chose to concentrate mainly on coronary heart disease, reperfusion injury, and organ storage for transplantation. They provided ample evidence for free radicals having a major contribution to these conditions and insight on how antioxidant therapy could be beneficial. However, as they point out, "only when the mechanisms and involvement of radicals in the pathogenesis of many disorders described in this review are understood will approaches to antioxidant therapies be designed effectively and targeted successfully." We agree with these comments and we think that they apply equally well to understanding how antioxidant defense affects injury by toxic chemicals. We hope that this review offers perspectives on the functions of cellular antioxidant defense that will prove useful to those investigating chemically induced tissue injury and free-radical-associated diseases.

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MOLECULAR METHODS FOR VIRUS DETECTION

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Detection Methods Using Chemiluminescence

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- References

I. GENERAL INTRODUCTION

Chemiluminescence, the emission of light from a chemical reaction, has been studied extensively for many decades. Chemiluminescent processes constitute a very special class of chemical reactions in which products (or intermediates) are produced in electronically excited states that are very short-lived and rapidly decay with concomitant emission of light. Similar chemiluminescent reactions, called bioluminescence, occur in nature in species as diverse as the firefly (*Photinus pyralis*), marine bacteria (*Vibrio harveyi*), and others. Most chemiluminescence reactions involve oxidations of

a variety of organic compounds as well as naturally occurring materials, resulting in the generation of light-emitting excited states. This phenomenon was first described with synthetic organic compounds in 1877 (Radziszewski, 1877).

Chemiluminescent reactions do not produce very high intensity light signals because of many efficient quenching processes that compete with the radiative decay of the excited states. Nevertheless, chemiluminescence has been used effectively as a very sensitive detection system in many applications (Carter and Kricka, 1982; Harber, 1982; Kricka and Carter, 1982), largely because no background light signals are generated since the emitting excited state is created in a dark chemical reaction (compared to scattered excitation light in fluorescence.) Therefore, in theory, every photon detected is a true signal of the assay. This feature of chemiluminescent molecules—coupled with long shelf-life, elimination of hazards associated with the use of radioisotopes, and their detectability at 10^{-21} moles (detection of alkaline phosphatase with chemiluminescent dioxetane substrate; Kricka, 1992)—makes them ideal as a reporter system for immunoassays and DNA probe hybridization assays.

In this chapter we describe the use of various chemiluminescence methodologies for the detection of viruses in DNA hybridization assays. A short discussion of instrumentation used in chemiluminescence measurement is also included.

II. CHEMILUMINESCENCE METHODS

A. Dioxetanes

Dioxetanes are four-membered cyclic peroxides that have been implicated as short-lived unstable intermediates in oxidation reactions that result in chemiluminescence (McCapra, 1966). Thus, 1,2-dioxetanes differ from most other chemiluminescence systems because these compounds do not require oxidation to emit light. Recently developed 1,2-dioxetanes that can be activated to luminesce by enzymes have been used successfully for bio-analyte detection. Dephosphorylation of adamantyl- and derivatized adamantyl-1,2-dioxetane phosphate substrates, such as AMPPD® [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate] and CSPD® [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate] by alkaline phosphatase results in the formation of a destabilized anion that fragments further to form an excited state of methyl *meta*-oxybenzoate anion that emits light at

477 nm (Fig. 1; Bronstein *et al.*, 1989a,1991; Bronstein and Dimond, 1990; Bronstein and Sparks, 1992).

1,2-Dioxetane substrates for alkaline phosphatase are widely used in DNA hybridization assays (Bronstein, *et al.*, 1990; Pollard-Knight *et al.*, 1990b; Tumolo *et al.*, 1992). DNA probes are labeled with alkaline phosphatase either indirectly, with a biotin or hapten label followed by binding streptavidin- or antibody-alkaline phosphatase conjugates, or directly by covalent bonding to enzyme (oligonucleotide probes). Biotin has been the most popular ligand for indirect labeling, but hapten labels other than biotin have also been employed including digoxigenin, fluorescein, and 2,4-dinitrophenyl. Dioxetane-based chemiluminescent indirect labeling and detection systems for DNA hybridization assays, as well as for immunoassays and DNA sequencing, are widely available from many commercial suppliers. With a nick-translated biotinylated DNA probe, as little as 380 fg (7.9×10^4 copies) of target pBR322 DNA can be detected on a Southern blot (Bronstein *et al.*, 1990). Using digoxigenin-labeled random-primed DNA probes with a membrane-based assay and photographic film detection, a sensitivity level of 10–50 fg of target DNA was obtained for the detection of purified cytomegalovirus (CMV) or parvovirus B19 DNA (Musiani *et al.*, 1991a).

Direct labeling of oligonucleotide probes with alkaline phosphatase (Jablonski *et al.*, 1986) is possible with systems from Promega (Madison,

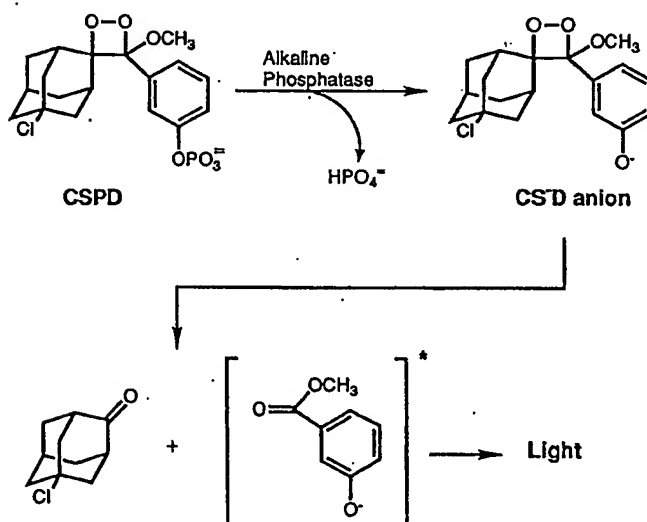


Figure 1 Chemiluminescent decomposition of CSPD® 1,2-dioxetane triggered by enzymatic dephosphorylation.

WI) and Cambridge Research Biochemicals (Wilmington, DE). The detection of a single copy gene in 0.25 μ g human genomic DNA with Southern blot analysis has been achieved with an alkaline phosphatase-labeled oligonucleotide probe (Cate *et al.*, 1991).

Comparisons have shown that the sensitivity of alkaline phosphatase-dioxetane chemiluminescence detection is comparable to or better than that of 32 P-based detection. In a human genomic Southern blot analysis of the tissue plasminogen activator gene, the sensitivity achieved with an alkaline phosphatase-labeled oligonucleotide probe was 12-fold higher than that achieved with the same 32 P-5'-end-labeled probe, and the speed of detection was enhanced 40-fold with the alkaline phosphatase-labeled probe (Cate *et al.*, 1991). Slot blot hybridization of human serum samples with the alkaline phosphatase-labeled AmpliProbe® system (ImClone Systems, New York) showed a higher sensitivity in the detection of hepatitis B virus (HBV) relative to 32 P-labeled nick-translated probes (Yang *et al.*, 1991). Similar sensitivities were obtained with an indirect digoxigenin-labeled probe and a random-primed 32 P-labeled probe in a dot blot hybridization assay for amplified human immunodeficiency virus type 1 (HIV-1) DNA (Zachar *et al.*, 1991).

The sensitivity of alkaline phosphatase-dioxetane chemiluminescence detection has been shown to be superior to other nonisotopic systems based on colorimetric detection in membrane-based hybridization assays (Bronstein and Kricka, 1989; Bronstein and Voyta, 1989; Bronstein *et al.*, 1989c; Musiani *et al.*, 1991a, 1992). Finally, alkaline phosphatase-dioxetane detection has been demonstrated to be two to five times more sensitive than enhanced luminol chemiluminescent detection (described subsequently) in a solution hybridization assay system (Clyne *et al.*, 1989; Urdea *et al.*, 1990). Furthermore, the alkaline phosphatase-dioxetane detection system consists of fewer components, which are more stable than those required for an enhanced luminol chemiluminescent reaction (Beck and Köster, 1990). Although several other alkaline phosphatase-based chemiluminescent assays also exist, involving alternative substrates and coupled reactions, the most sensitive and widely used assays are those with 1,2-dioxetane substrates (for review of alternative systems, see Kricka, 1991).

B. Luminol

Luminol and other cyclic diacylhydrazide derivatives can be oxidized in the presence of peroxide and peroxidase to generate an unstable intermediate in the excited state that chemiluminesces. Luminols can be used as direct chemiluminescent labels or as the chemiluminescent detectors of a peroxidase enzyme label (Kricka, 1991). Activation of luminol chemiluminescence with horseradish peroxidase (HRP) using an enhanced luminol system (en-

hanced chemiluminescence, ECL) has been done in DNA hybridization assays (Matthews *et al.*, 1985; Durrant *et al.*, 1990; Durrant, 1992) and immunoassays (reviewed by Bronstein and Sparks, 1992; Whitehead *et al.*, 1983; Thorpe *et al.*, 1985; Kricka *et al.*, 1987).

DNA probes can be labeled indirectly with HRP by binding streptavidin-HRP or anti-hapten-HRP conjugates or covalently by direct enzyme conjugation with oligonucleotides and longer double-stranded DNAs. The detection of single copy genes in 0.5 μ g human genomic DNA has been reported with indirectly labeled probes (Simmonds *et al.*, 1991). Direct HRP-labeled DNA probes have been used for both membrane-based DNA hybridization assays (Pollard-Knight *et al.*, 1990a; Simmonds *et al.*, 1991) and solution-phase hybridization assays (Urdea *et al.*, 1990). Detection of a single-copy gene on a Southern blot of <2 μ g human genomic DNA, with a sensitivity of <1 amol target DNA, has been demonstrated (Pollard-Knight *et al.*, 1990a) using direct HRP-labeled probes 0.3–5.1 kb in length. Similar sensitivity (1 amol target DNA) was also reported by Durrant *et al.* (1990). Both indirect and direct HRP labeling systems for nucleic acids and detection systems for HRP-catalyzed chemiluminescent reactions (ECL gene detection system) are available from Amersham (Arlington Heights, IL).

C. Acridinium Esters

Acridinium esters (AE) are direct chemiluminescent labels for antibodies (Weeks *et al.*, 1983) and DNA probes (Septak, 1989; Nelson and Kacian, 1990; Nelson *et al.*, 1992), in contrast to dioxetane and luminol systems, in which an enzyme label catalyzes the chemiluminescent reaction. *N*-Methyl acridinium esters react with hydrogen peroxide under basic conditions to yield an excited state *N*-methylacridone which emits light at 430 nm (reviewed by Nelson and Kacian, 1990). Oligonucleotide DNA probes can be labeled covalently with AEs by reaction of modified *N*-hydroxysuccinimide-AE with a primary alkyl amine on a linker arm that was previously incorporated during oligonucleotide synthesis (Nelson and Kacian, 1990). Preparation of AE-labeled oligonucleotide probes has also been described by Septak (1989). The AE label does not affect probe hybridization characteristics; relatively large amounts of clinical specimen material may be used without interfering with hybridization and detection of AE-labeled probes.

Probe hybridization and detection reactions are performed in solution, using either separation or nonseparation formats. In a separation or heterogeneous assay, hybridized probe may be separated and detected by selective binding to microspheres, which can be separated from solution magnetically. In a nonseparation or homogeneous format, also termed a hybridization protection assay (HPA), the ester bond of the unhybridized probe can be hydrolyzed by differential chemical hydrolysis, thus rendering its AE label

nonchemiluminescent, whereas the AE label of the hybridized probe is minimally affected (Nelson and Kacian, 1990). This type of assay is possible because hybridization provides an intercalation site for the AE label, thereby protecting the AE molecule residing in the hybridized region from hydrolysis (Arnold *et al.*, 1989).

The sensitivity of this detection system is approximately 5×10^{-19} mol AE-labeled oligonucleotide, and the linear dynamic range is greater than four orders of magnitude (Nelson and Kacian, 1990). Similar sensitivities for the detection of an amplified *gag* sequence (4 HIV proviral copies per 150,000 cells) were achieved with colorimetric, chemiluminescence and ^{32}P -labeling methods (Ou *et al.*, 1990; Rapier *et al.*, 1993). Schmidt (1991) was able to detect 0.05 fmol target HIV-1 DNA with AE-labeled *gag* probes and obtained greater sensitivity with chemiluminescence than with the same ^{32}P -end-labeled probe in a dot blot hybridization assay.

D. Electrochemiluminescence

Electrochemiluminescence is a process in which the excited state products are generated via an electrochemical reaction (Faulkner and Glass, 1982). Electrochemiluminescence occurs when specific metal chelates such as ruthenium (II) tris(bipyridyl) $[\text{Ru}(\text{bpy})_3^{2+}]$, utilized as labels, undergo a series of chemical reactions at an electrode surface. Electrochemiluminescent labels for DNA hybridization assays have been utilized in a highly sensitive, simple, and versatile assay system. Oligonucleotide probes, synthesized with a free 5'-amino group, are readily labeled with $\text{Ru}(\text{bpy})_3^{2+}$ -NHS ester (Blackburn *et al.*, 1991; Kenten *et al.*, 1991). Alternatively, oligonucleotide probes may be labeled during synthesis by incorporating labeled phosphoramidites (Kenten *et al.*, 1992; DiCesare *et al.*, 1993).

Electrochemiluminescent labels are relatively small molecules (~ 1000 dalton) that are extremely stable and may be coupled to nucleic acids, haptens, or proteins without affecting immunoreactivity or hybridization characteristics. The dynamic range for detection of these labels has been reported to be over six orders of magnitude (Blackburn *et al.*, 1991). These advantages, compared with other nonisotopic detection methods, provide potential wide utility in automated nonradioactive clinical diagnostic assays, including both DNA hybridization and immunoassay formats. A disadvantage of electrochemiluminescence, however, is a need for specialized instrumentation that can induce generation of electrochemically-excited states coupled with sensitive light detection.

Blackburn *et al.* (1991) used electrochemiluminescence detection with a DNA probe assay to quantify polymerase chain reaction (PCR)-amplified HIV-1 *gag* sequences. Double-stranded biotinylated PCR product was captured on streptavidin-coated microparticles and treated with alkali.

$\text{Ru}(\text{bpy})_3^{2+}$ -labeled oligonucleotide probe was then hybridized to the particle-bound DNA, washed, and quantified. A linear response was generated over the range of 50 to 2000 gene copies, and the detection of less than 10 copies of the HIV-1 *gag* was attained. An automated system for electrochemiluminescence quantification of PCR products (QPCR System 5000; Perkin-Elmer Corporation, Norwalk, CT) has been developed (DiCesare *et al.*, 1993) and is used for detection of viral disease. This system provides detection limits of 10–200 amol and a linear dynamic range greater than three orders of magnitude. The system has been used for the detection of HIV-1 over a range of 3 to 10^6 copies of target DNA (Wages *et al.*, 1993).

Because of the electrogeneration of the emitting species, which requires contact of the metal chelate label with an electrode, it is difficult to envision that simple membrane-based blotting assays that can be imaged on film could be designed using electrochemiluminescence.

E. Bioluminescence

Bioluminescent reactions, a special class of chemiluminescent reactions that occur in nature and are catalyzed by a luciferase or photoproteins, offer an alternative method for luminescence detection of protein and DNA (Kricka, 1991). Two bioluminescent reaction systems have been used for DNA hybridization assays, both of which are coupled enzymatic reactions. One system, used for membrane-based DNA hybridization, couples the production of D-luciferin from D-luciferin-O-phosphate, catalyzed by alkaline phosphatase (as a direct or indirect label) and the oxidation of D-luciferin, catalyzed by firefly luciferase, with concomitant light emission (Hauber and Geiger, 1987, 1988; Hauber *et al.*, 1988, 1989; Geiger, 1992). The other system, used with both membrane-based and solution hybridization assays, couples reactions catalyzed by glucose-6-phosphate dehydrogenase (G6PDH), NAD(P)H: FMN oxidoreductase, and marine bacterial luciferase to produce the light (Balaguer *et al.*, 1989a,b, 1991a,b; Nicolas *et al.*, 1990, 1992). Although bioluminescence-based DNA detection systems have not become as widely used as chemiluminescence systems for DNA hybridization assays, they do offer another alternative for sensitive nonradioactive biomolecule detection.

III. INSTRUMENTATION FOR CHEMILUMINESCENCE ASSAYS

A wide spectrum of instruments is currently available for recording and quantifying chemiluminescent signal intensities. These instruments, known as luminometers, use a light detector that consists of a photomultiplier tube

in photon counting mode, positioned close to the light source (microtiter plate or tube) to maximize photon collection efficiency. Among commercially available luminometers, semi-automated tube instruments such as the AutoClinilumat LB952T (Berthold/EG&G, Wallac, Inc., Gaithersburg, MD) and microtiter plate readers such as the ML 1000 (Dynatech Laboratories, Chantilly, VA) are most popular (reviewed by Bronstein and Kricka, 1990; Stanley, 1992a,b,1993b).

Chemiluminescence signals originating from blotting experiments performed on membranes can be detected by imaging on X-ray or instant photographic films. These films offer simple, convenient, and inexpensive detectors of chemiluminescence that can be used successfully for qualitative determinations and some signal quantification. Camera luminometers that house instant photographic film are suitable for the detection of light emission from blots and microtiter plate wells, and are available from Amersham, Analytical Luminescence Laboratory (San Diego, CA), Dynatech Laboratories, and Tropix, Inc. (Bedford, MA).

Finally, photon-counting cameras are available and are most suitable for the detection and accurate quantification of low-light signals. This instrumentation usually consists of a light detector such as a silicon target, silicon diode array, or charge-coupled device (CCD) coupled to a lens system, a controller, and a digital image processor. Since most of these camera systems are capable of imaging in two dimensions, micro- and macroscopic luminescent specimens can be analyzed spatially and temporally. The Argus-100/CL (Hamamatsu Corporation, Photonic Microscopy, Inc., Oak Brook, IL) is a photon-counting imaging device that has been used in the detection of blotted proteins (Hauber *et al.*, 1988). The Star I CCD cooled camera system (Photometrics Ltd., Tucson, AZ) exhibits very low dark current background and a wide dynamic range and has been used successfully to detect protein and nucleic acid analytes in solution and on membranes (Martin and Bronstein, 1993, 1994.).

IV. CHEMILUMINESCENCE ASSAYS FOR VIRUS DETECTION

The combination of DNA hybridization assays with chemiluminescence detection methods has enabled the development of rapid, sensitive, quantitative, nonradioactive assays that are amenable to automation. DNA hybridization technology is becoming accepted as a reliable clinical laboratory technique for the identification of infectious organisms and has fueled the need for more rapid, sensitive, and automated assay formats. Culture assay methods are laborious, time-consuming, and costly, and sometimes impossi-



ble to use. Antigen-based detection assays including fluorescent antibody and immunoassay techniques, although faster and automatable, are often less sensitive than culture techniques. With the advent of technologies such as PCR, DNA probe methods offer rapid, easy, and highly sensitive assay formats. DNA hybridization assays using radioactive labels are sensitive and are easily quantified, but health, environmental, disposal, and cost concerns render these systems less than ideal as widely used clinical assays.

Chemiluminescence methods for the detection of viral agents as well as other microorganisms have become widely used (Table 1), and continued development will certainly expand their applications in research and clinical diagnostic tests. More traditional immunoassays have also been developed and used with chemiluminescence for the detection of various viral antigens and the assessment of immune status with respect to viruses (selected references in Table 1). A survey of commercially available products that incorporate chemiluminescence or bioluminescence techniques and reagents for specific assays and nonspecific detection systems is available (Stanley, 1993a,b).

A. DNA Hybridization Assay Formats

Several DNA hybridization assay formats including membrane-based, solution, and *in situ* hybridization have been coupled with chemiluminescence for the detection of viruses and other infectious agents. Membrane-based chemiluminescent hybridization assays have employed either 1,2-dioxetane substrates for alkaline phosphatase or the enhanced chemiluminescence reaction of luminol and HRP, and are imaged on X-ray or photographic films or imaged directly and quantified using a CCD camera system. Solution hybridization assays are performed with 1,2-dioxetanes, luminol, and AE labels, and the emitted light signal is measured in a luminometer. Electrochemiluminescent labels are also used for solution hybridization assays and are detected with an instrument combining an electrochemical flow cell, a potentiostat, and a photomultiplier tube. *In situ* hybridization has been performed using both 1,2-dioxetanes and enhanced luminol with either photographic film detection or a CCD camera system.

B. Chemiluminescence Detection Systems

1. Dioxetanes

Alkaline phosphatase-dioxetane chemiluminescence systems have been used in a wide variety of DNA hybridization assays for detection of infectious

TABLE 1
Selected Studies That Have Used Chemiluminescence to Detect Viruses, *Chlamydia trachomatis*, and Other Microorganisms

Agent	Assay ^a	Reference
Barley yellow dwarf virus	MH	Foily <i>et al.</i> (1992) (DX)
Bluetongue virus	PCR/H	Akita <i>et al.</i> (1993)
Bovine enteric coronavirus	MH	Collomb <i>et al.</i> (1992) (LU)
Bovine immunodeficiency-like virus	IA	Jacobs <i>et al.</i> (1992)
Bovine leukosis virus	IA	Millukiene <i>et al.</i> (1991)
Bursal disease virus	H	Akin <i>et al.</i> (1993) (AP)
Chicken anemia virus	PCR/MH	Tham and Stanislawek (1992a,b) (DX)
Cytomegalovirus	MH	Musiani <i>et al.</i> (1991a,1992) (DX); Yang <i>et al.</i> (1991) (DX)
Dengue virus	PCR/H	Henchal <i>et al.</i> (1991) (DX)
Enterovirus (poliovirus)	MH	Fuchs <i>et al.</i> (1993) (DX)
Epstein-Barr virus	MH	Yang <i>et al.</i> (1991) (DX)
	PCR/MH	Vlieger <i>et al.</i> (1992) (LU)
Feline infectious peritonitis virus	MH	Marínez and Weiss (1993) (AP)
Grapevine closterovirus	IA	Pollini <i>et al.</i> (1993) (LU)
Hepatitis B virus	MH	Bronstein <i>et al.</i> (1989c) (DX); Farmer and Castaneda (1991) (DX); Yang <i>et al.</i> (1991) (DX)
	PCR/MH	Escarceller <i>et al.</i> (1992) (DX)
	SH	Urdea <i>et al.</i> (1987,1990) (LU; LU, DX)
	IA	Khalil <i>et al.</i> (1991a,b) (AE); Bourveresse and Bourgeois (1992) (AP); Boxall (1992) (LU); McCartney <i>et al.</i> (1993) (LU)
Hepatitis C virus	IM	Ireland and Samuel (1989) (LU); Robertson <i>et al.</i> (1991) (AE); Weare <i>et al.</i> (1991) (AE)
	PCR/H	Geiger and Caselmann (1992)
	IA	Khalil <i>et al.</i> (1991b) (AE)
Herpes simplex virus	MH	Bronstein and Voyta (1989) (DX)
	ISH	Bronstein and Voyta (1989) (DX)
	PCR/H	Puchhammerstoekl <i>et al.</i> (1993) (AP)
	IA	Pronovost <i>et al.</i> (1981) (LU); Dalesio and Ashley (1992) (LU)
Human immunodeficiency virus	PCR/MH	Conway <i>et al.</i> (1990) (AP); Zachar <i>et al.</i> (1991) (DX)
	PCR/SH	Ou <i>et al.</i> (1990) (AE); Blackburn <i>et al.</i> (1991) (EL); Schmidt (1991) (AE); Schmidt and Gschnait (1991); Gudibande <i>et al.</i> (1992) (EL); Kanten <i>et al.</i> (1992) (EL); Suzuki <i>et al.</i> (1992) (DX); Rapier <i>et al.</i> (1993) (AE); Wages <i>et al.</i> (1993) (EL)
	PCR	Bettens <i>et al.</i> (1991) (DX)
	BH	Ishii and Ghosh (1993) (AP)

Human papilloma virus	ISH RT IA PCR/SH MH ISH	Bronstein <i>et al.</i> (1989b) (DX) Suzuki <i>et al.</i> (1993) (DX) Khalil <i>et al.</i> (1991b) (AE); Jacobs <i>et al.</i> (1992) Balaguer <i>et al.</i> (1991b) (BL); Kenten <i>et al.</i> (1991) (EL) Sarkar <i>et al.</i> (1993) Hawkins and Cumming (1990) (LU)
Human T-cell leukemia virus	IA IM	Khalil <i>et al.</i> (1991b) (AE) Kuroda <i>et al.</i> (1992) (LU)
Human T-cell lymphotropic virus	IA	Papsidero <i>et al.</i> (1992) (LU)
Influenza virus	MH IM	McKimm-Breschkin (1992) (DX) Arenkov <i>et al.</i> (1991)
Lentivirus	RT	Cook <i>et al.</i> (1992) (DX)
Parvovirus	MH IA	Musiani <i>et al.</i> (1991a,b) (DX) O'Neill and Coyle (1992) (LU)
Potato virus Y, potato spindle tuber viroid	MH	Welnicki and Hiruki (1993) (AP)
Potato, pome fruit viroid	MH	Podleckis <i>et al.</i> (1993) (AP)
Respiratory syncytial virus, rotavirus	IM	Hornsteth <i>et al.</i> (1988) (LU)
Rubella virus	IM	Chanteloup <i>et al.</i> (1992) (AP)
Varicella zoster virus	PCR/H	Eis-Hubinger <i>et al.</i> (1992) (AP)
Bacteria	MH ?	Gustafsson and Persing (1992) (LU) Daly <i>et al.</i> (1991)
<i>Chlamydia trachomatis</i>	SH	Clyne <i>et al.</i> (1989) (DX); Urdea <i>et al.</i> (1989) (DX); Gratton <i>et al.</i> (1990) (AE); Mercer <i>et al.</i> (1990) (AE); Iwen <i>et al.</i> (1991) (AE)
	IA	Neman-Simha <i>et al.</i> (1991); Dumornay <i>et al.</i> (1992) (AE); Jang <i>et al.</i> (1992) (AE); Scieux <i>et al.</i> (1992a,b) (AE)
Mycobacteria	SH SD/SH PCR/MH SH PCR/MH PCR/MH	Bull and Shanson (1992) (AE) Donahue <i>et al.</i> (1993) (DX) Sriharan and Barker (1991) (DX) Urdea <i>et al.</i> (1989) (DX); Viaspolder <i>et al.</i> (1993) (AE) Barker <i>et al.</i> (1992) (DX) Staiber <i>et al.</i> (1991) (LU)
<i>Neisseria gonorrhoeae</i>		
<i>Plasmodium falciparum</i>		
<i>Toxoplasma gondii</i>		

* Assay formats include: BH, bead-based hybridization; H, hybridization; IA, immunoassay; IM, immunity; ISH, *in situ* hybridization; MH, membrane-based hybridization; PCR, polymerase chain reaction; RT, reverse transcriptase; SD, strand displacement; SH, solution hybridization.

* Chemiluminescent (CL) methods employed (if known): AE, acridinium ester; AP, alkaline phosphatase (most likely with dioxetane substrate); BL, bioluminescence; DX, dioxetane; EL, electrochemiluminescence; ILU, isoluminol; LU, enhanced luminol.

agents. Membrane-based hybridization assays have been used for the detection of HBV (Bronstein *et al.*, 1989c; Yang *et al.*, 1991; Escarceller *et al.*, 1992), herpes simplex virus (HSV-1) (Bronstein and Voyta, 1989), CMV (Musiani *et al.*, 1991a, 1992; Yang *et al.*, 1991), HIV-1 (Zachar *et al.*, 1991), and other viral agents (Fouly *et al.*, 1992; Tham and Stanislawek, 1992a,b; Fuchs *et al.*, 1993). Solution hybridization assays include those for HBV (Urdea *et al.*, 1990), HIV-1 (Suzuki *et al.*, 1992), and *Chlamydia* (Clyne *et al.*, 1989; Urdea *et al.*, 1989). *In situ* hybridization assays have been performed with both HSV-1 infected cells (Bronstein and Voyta, 1989) and HIV-infected cells (Bronstein *et al.*, 1989b). Finally, assays for retroviruses based on the detection of reverse transcriptase activity can be coupled with chemiluminescence detection by measuring the enzymatic incorporation of digoxigenin-labeled nucleotides with anti-digoxigenin alkaline phosphatase and a dioxetane substrate (Suzuki *et al.*, 1993).

Commercially available detection systems incorporating dioxetanes include the AmpliProbe® system (ImClone Systems) for membrane-based hybridization assays for HBV, CMV, and EBV (Yang *et al.*, 1991), the Hybrid Capture™ System HBV DNA Assay (Murex Diagnostics Ltd., Kent, UK), and solution hybridization assay systems for *Chlamydia trachomatis* and HBV detection (Chiron Corporation; Clyne *et al.*, 1989; Urdea *et al.*, 1989, 1990).

2. Luminol

DNA hybridization assays using the ECL system with direct HRP-labeled probes include detection of bovine enteric coronavirus in a slot blot hybridization assay (Collomb *et al.*, 1992) and a solution-phase hybridization assay for HBV DNA (Urdea *et al.*, 1987, 1990). *In situ* hybridization for detection of human papillomavirus (HPV) type 16 has been performed with an indirect labeled probe (Hawkins and Cumming, 1990). ECL systems have also been used for the immunoassay detection of several viruses, including grapevine closterovirus (Pollini *et al.*, 1993) and parvovirus B 19 (O'Neill and Coyle, 1992).

3. Acridinium Esters

DNA hybridization assays incorporating AE-labeled probes have been developed for detection of several infectious agents from clinical samples, including *C. trachomatis*, *Neisseria gonorrhoeae*, fungal pathogens, mycobacteria, and several common bacterial pathogens (Nelson and Kacian, 1990). These assay systems, called PACE 2™ and ACCUPROBE™, are

available commercially through Gen-Probe, Inc. (San Diego, CA). The Gen-Probe system for screening for *Chlamydia* has been compared with both culture and nonculture antigen detection methods including enzyme immunoassays and immunofluorescent antibody tests (Gratton *et al.*, 1990; Mercer *et al.*, 1990; Iwen *et al.*, 1991). The PACE 2™ system can provide a rapid, reliable alternative to culture and immunoassay methods for the detection of *Chlamydia* from cervical samples (Iwen *et al.*, 1991). Solution hybridization (hybridization protection) assays with AE-labeled probes have been used for the detection of PCR-amplified HIV-1 DNA (Ou *et al.*, 1990; Schmidt, 1991; Rapier *et al.*, 1993).

In addition, AEs have also been used to label antibodies that have been incorporated into automated immunoassay formats for the detection of infectious agents and antibody screening from clinical samples (Khalil *et al.*, 1991a,b).

4. Electrochemiluminescence

Electrochemiluminescence detection has been used in both manual (Blackburn *et al.*, 1991; Gudibande *et al.*, 1992; Kenten *et al.*, 1992) and automated (QPCR System 5000; Wages *et al.*, 1993) post-PCR amplification DNA hybridization assays for the detection of HIV-1 and HPV (Kenten *et al.*, 1991).

5. Bioluminescence

Detection of asymmetric amplified papillomavirus sequences using solution-phase hybridization with a G6PDH-labeled oligonucleotide and solid-phase capture has been performed using a bioluminescence assay (Bala-guer *et al.*, 1991b).

V. CHEMILUMINESCENCE DETECTION PROTOCOLS

A. Hepatitis B Virus

Hepatitis B "core antigen" DNA, immobilized on nylon membrane, is hybridized with an alkaline phosphatase-labeled oligonucleotide probe. Hybridized probe is then detected with the 1,2-dioxetane substrate AMPPD (Bronstein *et al.*, 1989c).

1. Materials

Hepatitis B core antigen plasmid DNA and alkaline phosphatase-labeled probe, included in a SNAP® Hybridization System, and GeneScreen Plus™ nylon membrane were obtained from NEN/DuPont (Boston, MA). AMPPD and CSPD are from Tropix.

2. Target DNA Preparation and Probe Hybridization

HBV "core antigen" (HBVc) plasmid DNA (100 ng; 1.2×10^{10} copies) was dissolved in 25 μ l sterile deionized H₂O and serially diluted with 0.3 M NaOH to produce target DNA samples ranging in concentration from 4.88×10^3 to 0.98×10^8 copies/ μ l. Blots were prepared as described here:

1. Incubate diluted DNA samples at room temperature for 15 min to denature, and spot 1 μ l of each dilution onto dry membrane strips (1 \times 8 cm).
2. Rinse blots with 2 M NH₄OAc and then with 0.6 M NaCl, 0.08 M sodium citrate, pH 7.0.
3. Prehybridize with 3 ml hybridization buffer [0.75 M NaCl, 0.075 M sodium citrate (5X-SSC), 0.5% bovine serum, 0.5% polyvinylpyrrolidone, 1% sodium dodecyl sulfate (SDS), pH 7.0] for 15 min at 55°C.
4. Hybridize with hybridization buffer containing 1.0 nM alkaline phosphatase-labeled oligonucleotide probe for 30 min at 55°C.
5. Wash sequentially for 5 min each in:

1X SSC, pH 7.0, 1% SDS at room temperature

1X SSC, pH 7.0, 1% Triton X-100 at 55°C

1X SSC, pH 7.0, at room temperature

3. Chemiluminescence Detection

1. Wash hybridized blots with 0.1% bovine serum albumin (BSA), 0.05 M sodium carbonate, pH 9.5.
2. Saturate blot with 100 μ l 1.6 mM AMPPD in 0.1% BSA, 0.05 M sodium carbonate, 1.0 mM MgCl₂, pH 9.5.

NOTE: Alternatively, an improved buffer (0.1 M diethanolamine, 1.0 mM MgCl₂, pH 10.0) can be substituted for this wash, using 0.25 mM AMPPD or CSPD in this buffer for substrate incubation.

3. Place blots in a plastic pouch and image light emission in a camera

luminometer with Polaroid Instant Black and White Type 612 (ASA 20,000) photographic film.

NOTE: Alternatively, blots can be imaged on standard X-ray film.

4. Digitize photographic film image using a black and white RBP Reflectance Densitometer (Tobias Associates, Inc., Ivyland, PA).

4. Results

Figure 2 shows a time course of the chemiluminescent DNA hybridization assay for HBVc antigen DNA. Serial dilutions of plasmid DNA were hybridized with alkaline phosphatase-labeled oligonucleotide probe, incubated with chemiluminescent substrate, and imaged on photographic film. Each photograph corresponds to a 30-min exposure. With this chemiluminescence assay, 1.18×10^6 copies of HBVc DNA can be detected within 30 min of substrate incubation. After a 2-hr incubation, 4.39×10^4 copies can be detected. In contrast, with the colorimetric bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate system, 9.8×10^7 and 1.07×10^7 copies can be detected after 30 min or 2 hr of substrate incubation,

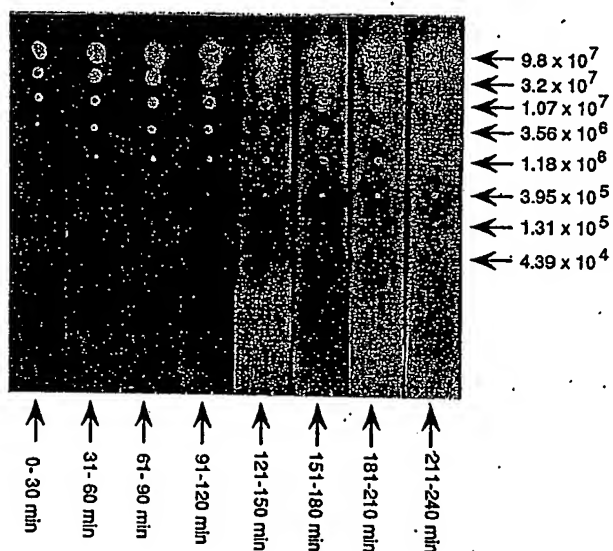


Figure 2 Chemiluminescent detection of hepatitis B "core antigen" plasmid DNA with AMPPD substrate in alkaline phosphatase-based DNA hybridization assay. Reprinted with permission from Bronstein *et al.* (1989c).

respectively (results not shown). Quantitative results were obtained by measuring reflection densities from the imaged photographic film using a black and white reflection densitometer (Fig. 3). These values could be used to establish a dose-response curve for the reflection densities as a function of HBVc plasmid concentration, from which HBVc DNA levels in clinical specimens could be determined. Use of the improved chemiluminescence detection protocol, incorporating the diethanolamine substrate buffer and CSPD chemiluminescent substrate, results in even greater sensitivity for DNA hybridization assays and would increase the sensitivity of this HBV DNA assay. Imaging and quantification of this membrane-based assay with rapidly evolving CCD camera systems will likely provide even greater sensitivity and a greater linear dynamic range than that achieved with densitometry.

5. Summary

Chemiluminescent detection of HBV DNA has also been performed with the AmpliProbe® system (ImClone Systems). This signal amplification probe system incorporates multiple target-specific primary and multiple secondary probes, alkaline phosphatase-labeled oligonucleotides that hybridize to the primary probes, in a two-step hybridization system (Yang *et al.*, 1991). Chemiluminescence detection is performed with a dioxetane substrate. With

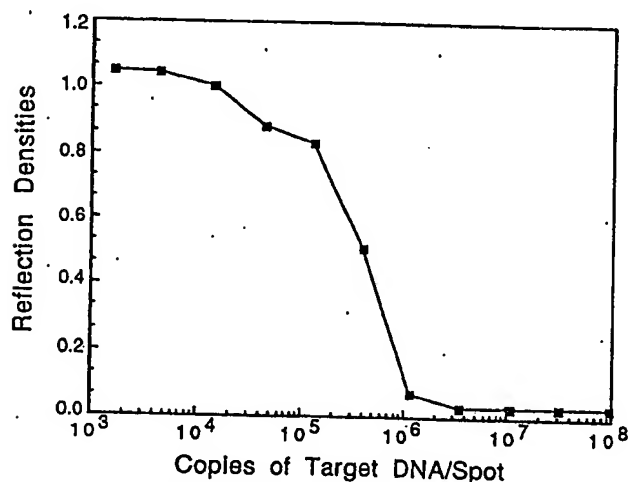


Figure 3 Hepatitis B virus "core antigen" plasmid DNA hybridization assay. Reflection density vs. number of copies of target DNA. Densitometric analysis of the Polaroid instant black and white photographic film image [0.00 (white)–2.00 (black)]. Reprinted with permission from Bronstein *et al.* (1989c).

this system, 0.4 pg (1×10^5 copies) purified target HBV genomic DNA can be detected in a chemiluminescent slot blot assay (Farmer and Castaneda, 1991; Yang *et al.*, 1991). Identical assays performed with serum samples (25 μ l) demonstrated that this chemiluminescent DNA hybridization system has the same specificity and sensitivity as immunoassays and is more sensitive than a 32 P-labeled nick-translated probe (Yang *et al.*, 1991).

Escarceller *et al.* (1992) report the use of digoxigenin-labeled probes, anti-digoxigenin alkaline phosphatase, and AMPPD for the direct detection of HBV sequences in human serum samples. These investigators achieved a limit of sensitivity of 2–5 pg, which was equivalent to that obtained with both colorimetric detection and a 32 P-labeled probe. These researchers also used digoxigenin-labeled oligonucleotide primers for PCR amplification of HBV DNA purified from human serum, followed by immunological detection of the digoxigenin label (as described), a method that can be used in conjunction with alternatively labeled primers for multiple amplifications.

A chemiluminescent assay incorporating a solution-phase hybridization of synthetic oligonucleotides to target DNA, followed by solid-phase capture, labeling, and detection with either HRP or alkaline phosphatase-labeled oligonucleotides and chemiluminescent substrates has been used to achieve the detection of 0.2 pg (6×10^4 copies) HBV DNA in human serum samples in 4 hr. This solution DNA hybridization method includes novel labeling and amplification schemes and has been performed with both polystyrene bead and microtiter well capture systems (Urdea *et al.*, 1987, 1990).

Chemiluminescence techniques have also been used in the development of automated enzyme immunoassay systems for the detection of HBV in human sera (Khalil *et al.*, 1991a,b; Bouveresse and Bourgeois, 1992).

B. Herpes Simplex Virus

Two chemiluminescent DNA hybridization assays for HSV, dot blot hybridization and *in situ* hybridization, are described here as originally reported by Bronstein and Voyta (1989). In these assays, HSV-1 plasmid DNA, immobilized on nylon membrane, or HSV-1-infected Vero cells, fixed and mounted on microscope slides, were hybridized with an alkaline phosphatase-labeled HSV-1 oligonucleotide probe and detected with AMPPD.

1. Materials

HSV-1 plasmid DNA and alkaline phosphatase-labeled oligonucleotide probe, included in a SNAP® Hybridization System, and GeneScreen Plus nylon membrane were obtained from NEN/DuPont. HSV-1-infected Vero

cells were provided by Drs. J. Kershner and E. Jablonski (Molecular Biosystems, San Diego, CA). AMPPD and Emerald™ luminescence-amplifying material are from Tropix.

2. Dot Blot Hybridization and Chemiluminescence Detection

This membrane hybridization protocol is similar to that described for HBV detection.

1. Serially dilute HSV-1 plasmid DNA in 0.3 M NaOH, denature, and spot 1- μ l aliquots onto dry membrane strips.
2. Prehybridize blots with hybridization buffer (0.5% BSA, 0.5% polyvinylpyrrolidone, 1% SDS) for 15 min at 55°C.
3. Hybridize with hybridization solution (containing alkaline phosphatase-labeled HSV-1 oligonucleotide probe) for 30 min at 55°C.
4. Wash sequentially for 5 min each in:

2X SSC, 1% SDS at room temperature

1X SSC, 1% Triton X-100 at 55°C

1X SSC, 1% Triton X-100 at room temperature

1X SSC at room temperature

5. Wash hybridized blots with 0.05 M sodium carbonate/bicarbonate, 1 mM MgCl₂, pH 9.5 (substrate buffer).
6. Saturate blot with 1.6 mM AMPPD (in substrate buffer) for 5 min.

NOTE: As described for HBV detection, the diethanolamine buffer and 0.25 mM AMPPD or CSPD can be substituted in Steps 5 and 6 for increased sensitivity.

7. Image blots with Polaroid Type 612 Instant Black and White film.

NOTE: Alternatively, blots can be imaged on X-ray film.

3. In Situ Hybridization and Chemiluminescence Detection

1. Infect Vero cells with HSV-1 (MacIntyre strain) for 1 hr at room temperature.
2. Harvest cells with trypsin/versene after the addition of 2% fetal calf serum at 0, 2, 4, 6, 8, 10, 12, 24, and 48 hr.
3. Pellet cells, fix in 95% ethanol, and mount on glass microscope slides.

4. Treat mounted slides with 0.2 M HCl for 2 min, rinse with deionized water, and immerse in 70% ethanol. Prior to hybridization, remove slides from ethanol and dry.
5. Immerse slides in 0.1% BSA, 5X SSC for 15 min at 70°C. Treat with 0.3 M NaOH for 1 min at room temperature. Rinse with phosphate-buffered saline (PBS).
6. Hybridize cells with the alkaline phosphatase-labeled HSV-1 oligonucleotide probe at a concentration of 5 nM in 0.1% BSA, 5X SSC for 20 min at 60°C.
7. Wash slides briefly in hybridization buffer at 60°C, and then extensively with 1X SSC at 50°C.
8. Wash with 0.05 M sodium carbonate/bicarbonate, 1 mM MgCl₂, pH 9.5 (substrate buffer).
9. Incubate with 0.8 mM AMPPD, 10% Emerald in substrate buffer for 5 min.
10. Place slides in a camera luminometer and expose to Polaroid Type 612 Instant Black and White film.

4. Results

With the dot blot hybridization assay for HSV-1 plasmid DNA, detection limits achieved with the chemiluminescent substrate AMPPD are 1.3×10^5 and 1.4×10^4 copies of target HSV-1 DNA, with a 30-min exposure performed 1 hr after substrate addition and a 45-min exposure performed 4 hr after substrate addition, respectively (results not shown). The sensitivity achieved with AMPPD is 25- to 100-fold higher than that obtained with the colorimetric BCIP/NBT substrate system (results not shown). Fig. 4 shows the time course of viral infection assayed by *in situ* DNA hybridization with chemiluminescence detection. Use of the AMPPD chemiluminescent substrate enables the detection of HSV-1-infected cells within 6 hr postinfection. Again, with this assay format, CCD detection and imaging may provide even greater sensitivity than that achieved with photographic film.

5. Summary

In situ hybridization with chemiluminescence detection has also been used to detect HIV-infected cells (Bronstein *et al.*, 1989b) and HPV type 16 in a cervical carcinoma cell line (Hawkins and Cumming, 1990). The latter protocol involved the use of biotinylated HPV 16 DNA probes (Enzo Diagnostics, New York), a streptavidin-HRP conjugate, and ECL detection re-

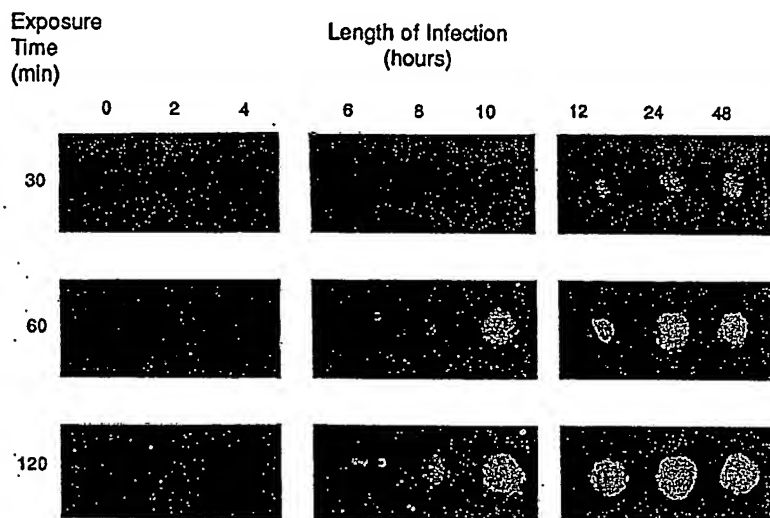


Figure 4 Chemiluminescent detection of *in situ* DNA hybridization of herpes simplex virus 1-infected Vero cells: time course of infection. Reprinted from Bronstein and Voyta, *Clinical Chemistry* (1989), 35, 1856-1857, Courtesy of the American Association for Clinical Chemistry, Inc.

agents coupled with a CCD imaging system. Detection of fewer than 10 HPV-positive cells (containing 600 copies of HPV 16 DNA per cell) among 10,000 HPV-negative cells on a single slide was achieved. However, this detection level is not necessarily the limit of sensitivity; with improved optical instrumentation, *in situ* hybridization coupled with CCD detection may provide a valuable diagnostic tool for the rapid and automated identification of viral sequences within cells.

VI. CONCLUSION

Chemiluminescence detection technologies combined with DNA hybridization methods provide rapid, sensitive, nonradioactive, automatable assay formats for the clinical diagnosis of infectious agents, as well as for research use. Rapidly evolving chemiluminescent enzyme substrates and labels, techniques, and assay and detection instrumentation, coupled with continued advances in DNA hybridization technologies, will further refine and improve the specificity and sensitivity of chemiluminescent DNA detection methods, bringing them into more widespread use.

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Neurotoxicology *In Vitro*

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8

Cell Contact and Cell Communication

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8.1 INTRODUCTION

Interaction and cooperation between cells in an organ allow for an organism to function as a coordinated living entity. There are several means by which cells can communicate with each other (Figure 8.1). Cells not directly in contact with each other or at distant sites from each other in the body can communicate through hormonal interaction via the blood. This hormonal regulation is mediated by the specific receptors unique to the target cell. Locally, cells also communicate through cell surface glycoproteins or adhesion molecules such as cadherins, integrins, or members of the immunoglobulin superfamily and thereby exchange information between cells and the extracellular matrix (Bell, 1978; Jessell, 1988; Hynes, 1992). Another important form of communication between two adjacent cells is through the gap junction, which is a surface organelle (Revel *et al.*, 1967) composed of two hemichannels (connexons), one from each cell. Gap junctions function as a conduit for the exchange of small water-soluble molecules and electrotonic transmission between excitable cells. The substances that have been demonstrated to cross gap junctions include amino acids, sugars, nucleotides, and second messengers such as cAMP and calcium (Rieske *et al.*, 1975; Pitts and Sims, 1977; Tsien and Weingart, 1976; Cornell-Bell *et al.*, 1990). By allowing these information changes, gap junctions facilitate the synchronization of electronic and metabolic cooperation, which appears to be required for the correct functioning of the cells, including maintenance of homeostasis, normal embryogenesis, control of cell proliferation, and maintenance of cell differentiation (Caveney, 1985; Loewenstein, 1981; Rose and Rick, 1978; Chang *et al.*, 1987).

Due to its important role in normal cell physiology, disruption of gap junctional intercellular communication has been postulated to be associated with a variety of pathological and toxicological conditions and disease states including carcinogenesis (Klaunig and Ruch, 1990), teratogenesis (Warner *et al.*, 1984), neurotoxicity (Trosko *et al.*, 1987), reproductive dysfunction (Ye *et al.*, 1990), cardiovascular diseases (Kleber *et al.*, 1987), ischaemia (Smith *et al.*, 1991) and cholestasis (Traub *et al.*, 1983).

In recent years, additional interest has been seen in defining the role of cell-to-cell communication through gap junctions in the nervous system. The diversity of cell types in the nervous system impacts on the type and degree of intercellular communication seen. Through their intercellular interactions these diverse cell types form functional interacting compartments within the brain. The degree of intercellular communication within each cell population varies according to cell type, and there is evidence that communication also occurs between different cell types. It has been generally held that chemical transmission is the major mechanism by which neuronal cells interact. However, more recently, morphological and electrophysiological evidence suggests the existence of extensive interneuronal gap junctional interaction. Electrical coupling through gap junctions in the nervous system may constitute an important mode of neuronal intercellular communication and contribute significantly to the integrative properties of the neuronal

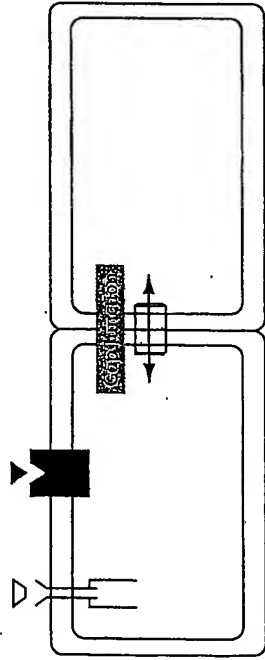


Figure 8.1: Modalities of intercellular communication. Hormones (∇) and growth factors (∇) are transported to the targets via blood. Ions and small molecules (<1000 Da) can pass through gap junctions from one cell to its neighbours and influence the function of the connecting cells.

cells. It is speculated that cell-to-cell coupling in the immature neocortex defines an important microenvironment to guide the formation of neuronal synapse (Peinado *et al.*, 1993). When it is maturing, gap junctional intercellular communication, as indicated by dye coupling, declines in neocortex, thus correlating inversely with the differentiation of the brain (Peinado *et al.*, 1993; Connors *et al.*, 1983). The possible roles of gap junctional intercellular communication between mature neurons have been discussed previously by Dermietzel and Spray (1993). Compared with neurons, gap junctions are more common in glial cells, especially in astrocytes. The extensive distribution of gap junctions and the resulting gap junctional intercellular communication seen between astrocytes give rise to an astrocytic syncytium which appears to function in the rapid redistribution of potassium ions after neuronal firing (Gardner-Medwin, 1983). The transfer and distribution of calcium and inositol triphosphate have also been suggested as a function of astrocytic gap junctional intercellular communication (Cornell-Bell *et al.*, 1990; Kim *et al.*, 1994). In C6 glioma cells gap junctions may propagate and amplify ischemic injury (Lin *et al.*, 1998). It can be concluded that gap junction mediated intercellular communication is an important means of buffering electrolytes and maintaining homeostasis in the astrocyte cell population in the CNS.

Its ubiquitous nature and important physiological role make the gap junction a target for the toxic effects of exogenous chemicals. It is of great help to understand the mechanisms of gap junction regulation before discussion of toxic effects. Several molecular intracellular steps must take place before gap junctions can be formed and function (Figure 8.2). First, mRNAs for different connexins are transcribed in the nucleus, and transported to cytoplasm for translation. Connexin protein is produced in rough endoplasmic reticulum and assembled in the Golgi. Six connexin proteins constituting a hemi-connexon structure are in turn inserted into the plasma membrane. Two hemi-connexons from adjacent cells are connected to form a channel. A collection of these conduits results in the formation of the gap junction membrane structure (Revel and Karnovsky, 1967). The correct orientation of the two hemichannels appears to be influenced by adhesion molecule mediated

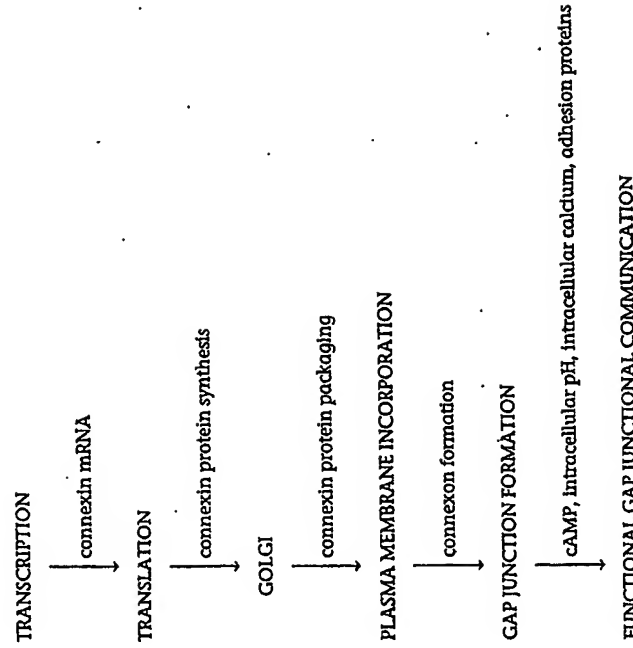


Figure 8.2: Molecular and intracellular control of gap junction formation and function

cell contacts (Kanno *et al.*, 1984; Mege *et al.*, 1988). After being successfully assembled, the function (intercellular communication) of the gap junction is dependent on the regulation and intercellular concentration of a variety of intracellular factors, including protein kinase C (Gainer and Murray, 1985; Hartman and Rosen, 1985), pH (Spray *et al.*, 1986), cAMP (Flagg-Newton *et al.*, 1981; Klaunig and Ruch, 1987) and intracellular calcium (Peracchia and Peracchia, 1980; Rose and Rick, 1978). Modulation of these factors affect the opening and closing of the gap junction channels and thus regulate intercellular communication. Toxic agents may target any of the stages of gap junction formation from connexin transcription, to protein synthesis, to assembly of the connexon in the plasma membrane, to the modulation of intracellular control of the pore opening and closing.

Since the first discovery of an electron microscopically detected structure, the nexus, extensive literature has appeared describing the structure, function and pathological changes of gap junctions. Along with this is the development of a variety of approaches to investigate these phenomena. Initial studies used electron microscopy and provided important structural information on the gap junction plaque and its components. Robertson (1963) described hexagonally packed subunits in electrical synapses between Mauthner cells in the medulla oblongata of the goldfish brain. Freeze-fracture electron microscopy has further described the relationship of connexin and connexon structures

(Rash and Yasumura, 1992). Revel and Karnovsky (1967) found an intercellular space in heart and liver that measured 1–2 nm in width and allowed tracer molecules to penetrate between cells. In excitable cells such as neurons, myocytes and smooth muscle cells, electrical coupling has been used to assess the function of gap junctions (Bennett and Goodenough, 1978). Metabolic cooperation and fluorescent dye transfer (dye coupling) have been extensively used to assess the functional nature of gap junctions. More recently, antibodies produced against connexin protein have been used to identify gap junction location and concentration in cell by immunocytochemistry (Shiosaka *et al.*, 1989; Yamamoto *et al.*, 1989). Similarly, *in situ* hybridization has been employed to define those cells that produce mRNA for specific gap junctions within a tissue and organ (Matsumoto *et al.*, 1991). While electron microscopy, immunocytochemistry, and *in situ* hybridization can be used to examine the structure and the distribution of gap junction proteins and cells making specific gap junctional proteins, these techniques provide little information of the functionality of the gap junctions in a tissue or organ. Functional assessment of the gap junction, intercellular communication, has been accomplished using electrical coupling, dye transfer, and metabolic cooperation.

8.2 USE OF CELL CULTURE AND TISSUE SLICE PREPARATIONS FOR THE ASSESSMENT OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

The examination of gap junctional intercellular communication in a normal situation is difficult, if not impossible. Even though the effects of toxicants on the expression of gap junction mRNA and proteins can be assessed following *in vivo* treatment using molecular and morphologic approaches, little information about the function of gap junctions (intercellular communication) has been retrieved by such studies. Several approaches have been described for the *in vitro* study of gap junctional intercellular communication in the nervous system. These methods basically fall into two types of preparation: tissue slice and cell culture (either primary or secondary). Both dye transfer (coupling) and electrophysiological coupling have been studied in these preparations.

In cell cultures, either primary or secondary, the effect of a compound on gap junctional intercellular communication can be assessed directly by measuring electrophysiological coupling or dye coupling following treatment of the cells with the chemical of interest. Use of primary and secondary cell cultures has provided important information on the mechanisms of action of toxic agents on neural cell gap junctions. In addition, the use of cell cultures provide a relatively easy and repeatable system by which multiple studies can be performed efficiently while also reducing the number of experimental animals involved. However, in culture, cells are more accessible to the toxicant due to the continuous bathing of the cells with the compound and they also lack the three-dimensional

structure seen *in vivo*. Thus, while cell culture approaches provide important information on the mechanisms involved in the interruption of cell-to-cell communication by toxic agents, the exact interaction of toxicants with the brain cells seen *in vivo* cannot be duplicated using a two-dimensional culture approach. In addition, when preparing and using cell cultures for gap junctional communication studies, modification of gap junction expression may occur during the isolation and culture process. This is supported in part by the fact that the incidence of dye coupling in neurons is lower in culture than in the hippocampal slice preparations (20 vs 50 per cent) (O'Beirne *et al.*, 1987).

Compared with cell cultures, tissue slice preparations have several advantages including that the normal three-dimensional intercellular contact and interaction seen in the brain are more closely maintained and the effect of *in vivo* treatment on the brain gap junctions can be more accurately determined. Tissue slice preparations also have disadvantages including a restriction in the number of replicates and various experimental manipulations (dose response) that can be performed in a timely manner. In addition, the evaluation of cell-to-cell communication using the slice techniques involves a greater amount of technical skill and effort.

8.3 DYE COUPLING VS ELECTROPHYSIOLOGICAL COUPLING

Dye coupling is the phenomenon of dye movement from one cell to an adjacent contacting neighbouring cell through the gap junction (connexon). The molecular weight cutoff for materials that traverse through the gap junction has been estimated to be less than 1000 Da for cells (Flagg-Newton *et al.*, 1979). Therefore, to assess gap junction function in mammalian cells using dye transfer (coupling), a molecular marker of less than 1 kDa must be used. In addition, probes for junctional permeability should have the following properties: strongly hydrophilic, impermeable through non-junctional membrane, low toxicity, and high sensitivity upon detection. The first fluorescent probe used to explore gap junction was fluorescein (Loewenstein and Kanno, 1964). However, this probe also displayed permeability through non-junctional membranes, which has prevented its further utilization. Other fluorescent dyes such as Procion yellow M4RS have been used in neuronal cell staining (Stretton and Kravitz, 1968). However, the low fluorescent efficiency of Procion yellow as well as its toxic effects in some cells has restricted its use (Payton *et al.*, 1969). In 1978, Stewart described and characterized a new fluorescent dye, Lucifer yellow CH. This dye has the properties noted above for use in gap junctional function evaluation. It is sensitive (intense fluorescence), has low toxicity, is not permeable through non-junctional membranes, rapidly spreads to the adjacent cells, and is retained during tissue fixation. These major advantages make Lucifer yellow CH a popular tool for testing gap junctional intercellular communication in a variety of cells, including neurons and astrocytes.

In our laboratory, Lucifer yellow CH has been used as a tracer for quantitative and qualitative evaluation of gap junctional intercellular communication in primary and

TABLE 8.1:

Effects of acrylonitrile (ACN) on gap junctional intercellular communication in rat astrocytes

Dose of ACN (M)	Duration of exposure (hours)		
	2	4	24
0	97.67 ± 1.4	94.64 ± 4.1	94.64 ± 4.0
1 × 10 ⁻⁵	94.34 ± 2.0	89.86 ± 2.5 ^a	84.63 ± 9.1 ^b
1 × 10 ⁻⁴	89.34 ± 1.6 ^a	76.29 ± 9.7 ^b	77.33 ± 18.2 ^b
1 × 10 ⁻³	83.18 ± 1.1 ^b	60.72 ± 6.2 ^b	63.43 ± 16.3 ^b
			70.90 ± 3.4 ^b

Values represent the mean ± the SD percentage of dye coupled astrocytes with $n > 3$.

^a Statistical significance from untreated control to $P < 0.01$ by Fisher exact test.

^b Statistical significance from untreated control to $P < 0.0001$ by Fisher exact test.

secondary cell cultures. Using Lucifer yellow, the effect of toxic agents on cell-to-cell communication can be examined in defined doses and time-dependent relationships following toxicant treatment. We have examined, for example, the effect of the neurocarcinogen, acrylonitrile, on gap junctional intercellular communication in a rat astrocyte cell line (DI TNC1 cell line). Acrylonitrile is a chemical monomer widely used in industry that induces astrocytomas in a dose response manner in rat brain following chronic exposure (Bigner *et al.*, 1986). Acrylonitrile appears to function through non-genotoxic carcinogenic mechanisms, possibly through the induction of oxidative damage (oxidative stress). Blockage of cell-to-cell communication appears to be a characteristic of most non-genotoxic carcinogens and has also been seen following exposure to reactive oxygen species. Acrylonitrile inhibited gap junctional intercellular communication in rat astrocytes in a dose-dependent manner (Table 8.1). This inhibition was prevented by the cotreatment with vitamin E, a well known antioxidant (Table 8.2). These data confirm a role of oxidative stress in acrylonitrile-induced inhibition of gap junctional intercellular communication in rat astrocytes, and correlate with previous *in vitro* and *in vivo* observations by our group which showed acrylonitrile-induced oxidative stress in both rat brain and rat astrocytes (J. Jiang and J.F. Klaunig, unpublished data).

Electrical coupling is the capacity for passive (electronic) spread of a transient electrical potential from a cell to an adjacent contiguous cell (Socolar and Loewenstein, 1979). When pulses of hyperpolarizing or hypopolarizing current are passed from cell to cell with the aid of intracellular microelectrodes, the detection of the voltage deflection in both the stimulated cell and the communicating neighbour cell can be made. This transduction of electrical coupling potential is bidirectional, and the gap junction has been demonstrated as the structural basis for this electrical transmission (Peracchia, 1980; Loewenstein, 1981). Although this method is sensitive in indicating gap junctional permeability, the procedure is extremely time-consuming and difficult (Rao *et al.*, 1986). The other problem

TABLE 8.2:

Effects of d- α -tocopherol (Vit E) on acrylonitrile (ACN) induced inhibition of gap junctional communication in rat astrocytes

Dose of ACN (M)	Duration of exposure (hours)			
	4	4 + 10 ⁻⁴ M Vit E	24	24 + 10 ⁻⁴ M Vit E
0	91.69 ± 5.2	94.38 ± 3.9	94.59 ± 5.0	95.97 ± 1.1
1 × 10 ⁻⁵	89.99 ± 3.4	89.84 ± 3.3	86.33 ± 5.0 ^a	89.21 ± 1.5
1 × 10 ⁻⁴	84.88 ± 0.9 ^a	86.03 ± 8.5	83.80 ± 2.3 ^c	84.61 ± 6.6 ^c
1 × 10 ⁻³	62.37 ± 5.8 ^{ac}	84.22 ± 10.1 ^{ab,d}	68.80 ± 4.5 ^c	84.55 ± 7.0 ^d

Values represent the mean ± the SD (% recovery) percentage of dye coupled astrocytes with $n > 3$.

^a Statistical significance from untreated control to $P < 0.05$ by Fisher exact test.

^b Statistical significance from respective treatment group to $P < 0.05$ by Fisher exact test.

^c Statistical significance from untreated control to $P < 0.01$ by Fisher exact test.

^d Statistical significance from respective treatment group to $P < 0.01$ by Fisher exact test.

encountered using electrophysiological methodology is the difficulty in selecting a coupled pair of neurons. Because the coupling is not always somatic to somatic, two neurons might be apart from each other but still connected through gap junctions in the dendrites. These technical problems make dye-coupling a more attractive approach for the effects of toxicants on nervous system gap junctional intercellular communication.

8.4 METHODOLOGICAL CONSIDERATIONS

A number of culture systems, discussed extensively in Chapter 10, are available for the analysis of intercellular communication via gap junctions. In the following sections we briefly summarize some specific procedures successfully employed in this laboratory.

8.4.1 Cell culture: rat hippocampal neurons and rat astrocyte cell line (DI TNC1)

Hippocampi from 18-day-old gestational rats are dissected, dissociated and cells plated onto polylysine-coated coverslips by the standard methods (see O'Beirne *et al.*, 1987). Coverslips with neurons are transferred to a recording chamber on an inverted microscope and are perfused with oxygenated (5% CO₂-95% O₂) artificial CSF (Knut and Westgaard, 1971). Neurons are easily recognized because their cell bodies are bright by phase contrast microscopy and are raised above the glial monolayer.

Rat astrocytes (DI TNC1) are plated at 1 × 10⁶ cells per 60 mm culture dish. Cells are cultured in DMEM/F12 medium containing 4.5 g/l glucose, 10% FBS and 10 ml of Pen/Strep solution. Cultures are incubated at 37°C and 5% CO₂ for 24 hours prior to treatment.

8.4.2 Loading and detection of fluorescent dye coupling

Loading dye into cells can be accomplished by several means, including microinjection (Stewart, 1978) and scrape-loading (El-Fouly *et al.*, 1987). Because it is easy to use and easy to control, microinjection with iontophoresis is the most popular technique in studying gap junctions in nervous system in both slice preparation and cell culture. We also focus on this method in this review (Klaunig and Baker, 1994).

Microelectrode injection needles are pulled from 1.5 mm diameter single-barrel glass capillaries. The tip opening diameter is measured through tip resistance to be 1 μ m. Using a binocular dissecting scope, the pipettes are initially loaded with 5 μ l of 5% Lucifer yellow CH and backfilled with LiCl. The injection pipettes must be made fresh before each injection period. The injection micropipette is located in the microscope field of view at 200 \times and manoeuvred slowly to the cell of interest using the micromanipulators until the tip pierces the plasma membrane at a glancing angle. A current of 3 nA is then applied into the micropipette, forcing the Lucifer yellow CH into the cell cytoplasm.

In primary culture, impalements are monitored throughout the injections to ensure that neurons remain excitable and membrane potential is stable. Live cells injected with Lucifer yellow are visualized in the recording chamber using epifluorescence and photographed immediately. The χ^2 test for homogeneity is used to compare the incidence of dye coupling between groups (O'Beirne *et al.*, 1987).

In secondary cell culture, the cell that is injected with Lucifer yellow (referred to as the donor cell) is viewed with the fluorescence microscope and the amount of dye coupling is scored. Dye coupling is quantitated by counting both the number of cells (recipients) that contact the donor cell that express dye coupling to them and the number of recipients that do not show dye coupling with the donor cell. The percentage of dye coupling is determined by dividing the number of coupled recipients by the total number of recipients (both coupled and uncoupled). Enough donor cells are microinjected so that 50–75 recipients can be evaluated per culture dish. Triplicate dishes should be examined for each concentration and duration of toxicant examined (Klaunig and Baker, 1994).

8.4.3 Electrophysiological coupling

Intracellular recordings are obtained in culture using conventional microelectrodes. Impalements are monitored throughout the injections to ensure that neurons remain excitable and membrane potential is stable. Simultaneous intracellular recordings are obtained from adjacent neurons to test for electronic coupling. The χ^2 test for homogeneity is used to compare the incidence of dye coupling between groups (O'Beirne *et al.*, 1987).

8.4.4 Tissue slices

8.4.4.i Preparation of tissue slices

Tissue slices have been successfully prepared from piriform cortex (Richards and Sercombe, 1968), the hippocampus (Schwartzkroin, 1975), neocortex (Gutnick and Prince, 1981). All of the existing procedures follow the same basic principles. The following procedure is based on the version of Yamamoto (1972). The region of interest is isolated from the rest of the brain and placed on a piece of filter paper covered with the standard medium. Sections of brain (about 0.3 mm thick) are prepared by cutting the tissue with a razor blade under binocular microscopic observation. Less than 5 minutes after the animal is killed the slices are placed in a constant-flow incubation chamber on a silk mesh in artificial cerebrospinal fluid (composed of NaCl, 134 mM, KCl, 5 mM, KH_2PO_4 , 1.24 mM, MgSO_4 , 1.3 mM, CaCl₂ 0.75 mM, NaHCO_3 , 16 mM, and glucose, 10mM) saturated with 95% of O_2 and 5% CO_2 . The same gas mixture is moistened and passed over the upper surface of the preparation.

8.4.4.ii Detection of dye coupling

Dye injection

The following method of detecting dye coupling in slices of brain is a modified version of MacVicar and Dudek's (1982). Microelectrode injection needles are pulled as described in section 8.4.2. Using a binocular dissecting scope, the pipettes are initially loaded with 5 μ l of 5% Lucifer yellow CH and backfilled with LiCl. Cells are injected with dye by passing constant-current hyperpolarizing pulses of 1 s duration at a rate of one every 2 s. During dye injection, action potentials of the cell are monitored. All recordings are obtained from cells with action potentials >50 mV. Only one cell is injected per slice. To minimize the extracellular concentrations of the stain, injections are immediately terminated when the penetration is lost or has significantly deteriorated. To control for artificial staining from Lucifer yellow leakage, neurons that have been briefly impaled but not iontophoresed are impaled for a varying duration. Only impalements for longer than 15–30 s result in detectable staining, and this staining is faint. Poor impalements obtained while searching for a stable recording are terminated in <10 s and Lucifer yellow leakage into these cells does not cause inadvertent staining.

Tissue fixation and recording

The fixative of choice is 4% formaldehyde, in 0.1 M sodium phosphate buffer (pH 7.4). This fixative gives good preservation of tissue and cell structure, and causes only a slight increase in background fluorescence. Five to 10 minutes after injection, the slices are placed in 5% formaldehyde in 0.1 M phosphate buffer for 12–24 hours. The slices are then transferred to buffer containing 30% sucrose. Fixed slices are sectioned at 40 μ m on a freezing microtome. Sections are placed on slides, cleared (xylene), and mounted in PDX

(a mixture of polystyrene and butyl phthalate in xylene) or methyl salicylate. The sections are then observed with a fluorescence microscope and photographed (MacVicar and Dudek, 1982).

8.4.4.iii Electrophysiological coupling

This method of assessing electronic transmission is based on that described by Knowles *et al.* (1982). The possible existence of electronic coupling between neurons is examined by simultaneous penetration of two neurons. In order to interpret the data, only healthy penetrations are considered for the final analysis. Criteria for healthy penetrations are: membrane potential greater than 50 mV; input resistance greater than 20 M Ω (measured with 0.5 nA \times 100 ms hyperpolarization intracellular current pulse); spike width less than 2 ms; a steady train of action potentials in response to a 0.5 nA \times 100 ms depolarizing intracellular current pulse. Intracellular penetrations of neurons are made using visual guidance to place the electrodes in slices for somatic penetration. After establishing a healthy penetration of one neuron, a second microelectrode is aimed as closely as possible at the tip of the first electrode to penetrate a second neuron within 50 μ m of the first. When two penetrations have been established, current pulses are injected into one neuron while the membrane potential of the other neuron is examined at high gain. Depolarizing pulses (0.5 nA \times 100 ms) are used to elicit a spike train in one neuron, while the membrane potential of the other neuron is carefully examined for evidence of postsynaptic potentials. Similarly, when hyperpolarizing pulses (0.5–1.0 nA \times 100 ms) are injected into the first cell, the membrane potential of the second cell is examined for signs of electronic coupling potentials. The procedure is then switched to test for connections in the opposite direction. The intracellular potentials are amplified using high input impedance, capacity compensated amplifiers with internal bridge circuits for injecting current into the cells while recording. The potentials are displayed on an oscilloscope and stored on FM tape for later analysis and photography. The sensitivity for detection of electronic coupling potentials is estimated to be 1 mV, which would allow detection of cells with coupling coefficients greater than about 0.05 (Knowles *et al.*, 1982).

8.5 CONCLUSIONS

Since the identification of the gap junction and its importance in normal cellular physiology, extensive efforts have been made to understand the structure, function, and the regulation of this important cytoplasmic organelle. These efforts have been accompanied by the development of new approaches and methodologies to assess gap junctional intercellular communication. In recent years the role that the gap junction plays in toxicologically induced disease has also received increased attention. The approaches outlined above (dye coupling in cell culture and tissue slices) allow for further evaluation of both normal and toxicant-modified gap junction function.

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Brain Spheroid and Other Organotypic Culture Systems in In Vitro Neurotoxicology

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Chapter 14

Oncogenes, Tumor Suppressor Genes, and Intercellular Communication in the Oncogeny as Partially Blocked Ontogeny Hypothesis

James E. Trosko, Chia-Cheng Chang, Burra V. Madhukar, and Emmanuel Dupont

Cancer is a disease of faulty gene expression that results in "partially blocked ontogeny" and an insensitivity to organismic needs.

V. R. Potter (1988)

INTRODUCTION

It has often been stated that in understanding cancer, one can understand normal cell growth. Equally important, however, is that in understanding normal cell growth and behavior, insight to the carcinogenic process will be gained. The insights provided by the quotation from Van R. Potter serve to highlight two major principles of higher organism biology that unfortunately, seem to have

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Chapter 14

Oncogenes, Tumor Suppressor Genes, and Intercellular Communication in the Oncogeny as Partially Blocked Ontogeny Hypothesis

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Cancer is a disease of faulty gene expression that results in "partially blocked ontogeny" and an insensitivity to organismic needs.

V. R. Potter (1988)

INTRODUCTION

It has often been stated that in understanding cancer, one can understand normal cell growth. Equally important, however, is that in understanding normal cell growth and behavior, insight to the carcinogenic process will be gained. The insights provided by the quotation from Van R. Potter serve to highlight two major principles of higher organism biology that unfortunately, seem to have

been ignored by modern reductionalistic cancer scientists, namely the "hierarchical" (Brody, 1973) and "homeostatic" or "cybernetic" (Potter, 1974; Iversen, 1965) principles.

The normal multicellular organism, starting from a single totipotent stem cell, the fertilized egg, ends up as an organized and orchestrated collection of pluripotent stem, progenitor, and differentiated cells. These cells are capable of contributing to either further growth, development, differentiation, wound healing, or adaptive responses before death ensues. In other words, the human being with all of its functions is not just a collection of 100 trillion individual cells, but an organized and orchestrated collection of interacting groups of different kinds of cells.

These concepts, derived over many years from investigators representing many disciplines (eg, Claude Bernard, W. B. Cannon, P. Weiss, J. L. Kavanagh, O. H. Iversen, E. E. Osgood, and V. R. Potter; see Iversen, 1965, Potter, 1981), postulated the existence of positive and negative regulatory factors that existed between stem/progenitor cells and their differentiated daughters to control growth and differentiation. Clearly, this implied that positive and negative factors altered the target cell's phenotype by some intracellular signaling process. In brief, the conceptual framework postulates that intercellular communication plays a major role in the regulation of cell growth, differentiation, and adaptive response of all the cells of a multicellular organism.

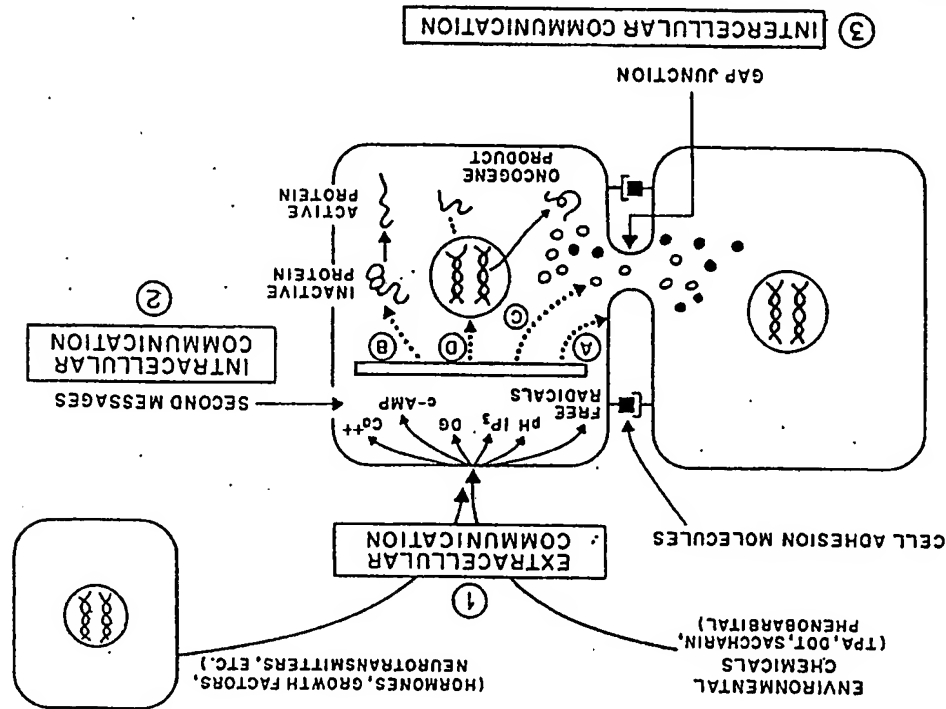
The general pathway for this cybernetic feedback system appears to be via the production of positive factors (Growth factors, hormones, and neurotransmitters, ie, extracellular positive signals) that trigger receptors, and transmembrane signaling elements (intracellular communicating molecules). These signals, in turn, are transferred to neighboring cells or modulate intercellular mechanisms within tissues (Figure 1).

After receiving these signals, the targeted cells alter their physiology in response to these signals, producing negative extracellular signals that feedback to the positive sources. If one accepts this basic concept of all normal higher organisms, then by logic alone one would reason that a blockage or breakdown or any one of these steps (extracellular, intracellular, or intercellular communication) should lead to the inability to control cell growth, differentiation, or adaptive responses of differentiated cells. This would seem to describe the cancer cell.

THEORIES OF CARCINOGENESIS

All scientific theories must explain observations in order that testable predictions can be made to falsify the theory. The major observations that are to be the focus of this analysis are: (a) normal cells are potentially contact inhibitable; cancer cells appear to be contact-insensitive (Borek & Sachs, 1966); (b) normal cells derived from stem and progenitor cells are capable of terminal differentiation; cancer cells, under their normal growth conditions, do not terminally differentiate (teratomas represent a special case; however, in principle, they do not break this

Figure 1 Schematic diagram characterizing the postulated link between extracellular and intercellular communication through various intracellular transmembrane signaling mechanisms. Intercellular communication alters membrane function, activates inactive proteins, modulates gap junction function, and modulates gene expression. It provides an integrated view of how the neuroendocrine-immune system (mind or brain/body connection) and other multisystem coordinations could occur. We do not show here, activation or altered expression of various oncogenes (antioncogenes) could also contribute to the regulation of gap junction function. Reproduced from Trosko J.E., Chang CC (1989). *Toxicology Letters*, 49, 283-295 with permission from Elsevier Science Publishers, Amsterdam, The Netherlands.



rule; Chang et al, 1990); and (c) most, if not all, tumors appear to be of clonal origin (Fialkow, 1979).

Several theories, based on these observations, have been proposed; cancer as a "disease of differentiation" (Markert, 1968; Pierce, 1974); initiation/promotion/progression theory of carcinogenesis (Pitot et al, 1981); and the oncogene/tumor suppressor gene theory of carcinogenesis (Weinberg, 1991). To integrate all of these different but overlapping theories, the theory of cancer as "dysfunctional intercellular communication," first postulated by Loewenstein (1966), was modified to integrate these other theories, as well as to incorporate recent observations related to gap junctional intercellular communication (Trosko et al, 1983; Trosko et al, in press).

In brief, the hypothesis to be developed here is that, starting with a normal stem or progenitor cell, stable alteration of a gene (an oncogene) that controls terminal differentiation but does not alter the control of cell proliferation (ie, contact inhibition or some form of gap junctional intercellular communication) would constitute the initiation phase of carcinogenesis. As long as that initiated stem or progenitor cell is communicating with other normal (heterologous) or initiated (homologous) cells, there will be no cell proliferation. On the other hand, if this gap junctional intercellular communication (GJIC) is inhibited by endogenous (growth factors or hormones) or by exogenous chemicals in a reversible fashion, then clonal expansion of these initiated cells can occur (tumor promotion). Because of their inability to terminally differentiate, these initiated cells, once stimulated to proliferate, will slowly accumulate as a benign monoclonal focus of nonterminally differentiated cells.

If other irreversible or mutagenic events occur during this process in a gene that stabilizes the blockage of intercellular communication (ie, in another oncogene or tumor suppressor gene), then the progression phase of carcinogenesis could occur (Figure 2).

This hypothesis has now integrated all of the aforementioned theories by means of postulating that a normal cell has the ability to communicate with other normal cells. In other words, initially a normal stem cell, through cell to cell extracellular communication, is regulated by its terminally differentiated daughter. The transition to a normal progenitor involves growth control by means of GJIC. If one of these progenitors is initiated, it will only proliferate, but not terminally differentiate, by either endogenous/exogenous reversible down-regulation of GJIC or by stable down-regulation by imbalance of oncogenes/tumor suppressor gene activities.

CELL-CELL COMMUNICATION IN NORMAL GROWTH CONTROL, DIFFERENTIATION, AND ADAPTIVE FUNCTIONS OF DIFFERENTIATED CELLS

The broad concept of homeostasis and cybernetics as it applies to regulatory control of cells during growth, development, differentiation, wound healing, and

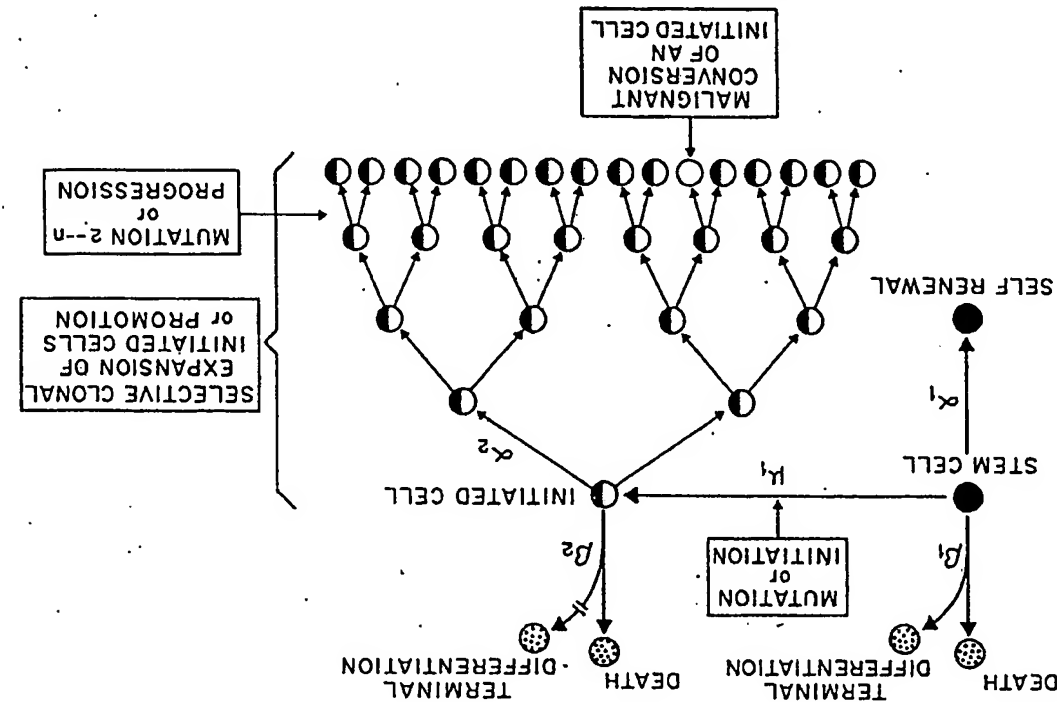


Figure 2. The initiation/promotion/progression model of carcinogenesis. β_1 = rate of terminal differentiation and death of stem cell; β_2 = rate of death, but not of terminal differentiation of the initiated cell (ie, possibly mutation); μ_1 = rate of the molecular event leading to initiation (ie, possibly mutation); μ_2 = rate at which second event occurs within an initiated cell. Reproduced from Trosko, JE et al (1988). *Gap Junctions*, edited by EL Hertzberg and RG Johnson with permission from Wiley-Liss, New York.

adaptive responses implies extracellular communication from one cell to another over a distance through hormones, growth regulators, neurotransmitters, and extracellular matrices. Intracellular communication includes all the second messages initiated by the extracellular signals. These second messages not only trigger changes intracellularly but can (a) spread to other cells by the intercellular gap junction channels (Saez et al, 1989) or (b) up- or down-regulate the gap junction channels (Spray & Bennett, 1985).

Intercellular communication is mediated by protein channels in membranes of contiguous cells (connexons). Each cell contributes a hemichannel composed of a hexamer of proteins (connexins). Clusters of these connexons allow ions and small molecules (below 1000 daltons) to freely equilibrate between coupled cells. There exists a family of highly conserved genes coding for these proteins (Fishman, Eddy, Shows, et al, 1991). From an evolutionary perspective, the gene for gap junctions appeared when multicellular organisms appeared (Revel, 1988).

Philosophically, the manner by which single-cell organisms (which are devoid of gap junctions) adaptively survived was by means of unlimited growth (contingent only on exogenous nutrients, etc). Multicellular organisms acquired differentiated functions for adaptive means to survive. The transition from unlimited growth to differentiation by a multicellular organism as means to survive coincided with the appearance of the gap junction (Revel, 1988). A multicellular organism needed to have a means to orchestrate the delicate balance of the cells' new roles. Namely, in addition to stimulate cell growth, the organism needed to control cell growth; it had to differentiate; it had to have renewal and wound healing potential; and once terminally differentiated it had to be able to adaptively change from a quiescent state to a functional state. While single cell organisms can communicate through extracellular means and respond to these signals with intracellular second messages, they lacked intercellular communication by means of gap junctions.

Most normal cells of a metazoan (except single mature red blood cells and normal stem cells; Chang et al, 1987; 1990) have gap junctions. These gap junctions appear to play roles of electrotonic synchronization in tissues such as heart or uterine muscle, of metabolic equilibration in nonexcitable tissues, of growth control in contact-inhibited premitotic cells, of differentiation control as in the maturation of sperm and eggs, and of adaptive response as in production of insulin in pancreatic cells (Hertzberg & Johnson, 1988).

When quiescent or contact-inhibited, premitotic cells' gap junctions are up-regulated and functional. When normal cells are stimulated by growth factors (Madhukar et al, 1989; Maldonado et al, 1988), mitogenic tumor promoters (Trosko & Chang, 1988), or wound-healing signals (Radu & Moldovan, 1991), there is a transient decrease or down-regulation of gap junctions. While it is important to determine if gap junctions need to be down-regulated before cell proliferation (cause) or whether the decrease in GJIC is the consequence of cell

proliferation, several recent observations seem to suggest that a decrease in GJIC precedes cell proliferation (Azamia et al, 1988; Shiba, 1991).

The picture that seems to be emerging is that during the development and adaptive functioning of a mature multicellular organism, cells of the different organs are interacting within and between tissues. Within the tissue, stem cells are interacting with their differentiated daughters (extracellular communication). Progenitor and differentiated cells are communicating by both extra- and intercellular means (de Rooij et al, 1985). In addition, homologous GJIC occurs between some cells (eg, liver epithelial cells couple with like-type cells; hepatocytes couple with hepatocytes; however, liver epithelial cells do not couple with hepatocytes, yet they seem to communicate through extracellular means; Mesnil et al, 1987). Other cells execute GJIC through heterologous means, such as Sertoli cells and spermatogonia (Gilula et al, 1976). Since these extracellular signals are known to modulate GJIC (Madhukar et al, 1989; Maldonado et al, 1988; Larsen, 1983), they could either stimulate cell proliferation (by down-regulation of GJIC in premitotic cells); cell differentiation (by either up- or down-regulation of GJIC in specific homo- or heterologous coupling situations); or differentiated functions (Meda et al, 1987).

DYSFUNCTIONAL GAP JUNCTIONAL INTERCELLULAR COMMUNICATION AND CANCER

If the foregoing discussion of the importance of GJIC is for the regulation of cell growth and differentiation is correct, then a single hypothesis can be postulated.

Postulate I. Normal premitotic cells are characterized by contact inhibition and the ability to terminally differentiate.

Postulate II. Gap junctional intercellular communication is necessary for growth control and terminal differentiation.

Postulate III. Cancer cells are characterized by the loss of growth control and by the inability to terminally differentiate.

Hypothesis. The transition from a normal stem/progenitor cell to a cancer cell must be due partly to its inability to perform GJIC.

Is there evidence to test this hypothesis? First, let it be clear that the hypothesis is based on the concept of gap junctional intercellular communication. In order that cells can perform GJIC: (a) they must be able to adhere (cell adhesion molecules must be functional; Jongen et al, 1991); (b) they must be able to couple their gap junctions; (c) they must be able to transfer ions and small molecules through the gap junctions; and (d) once into cells, these ions

and regulatory molecules must be able to elicit their response. If any of these steps is blocked, GJIC is blocked.

Most Normal Cells Have GJIC

To date, surveys using either electron micrographic analysis, functional coupling studies, antibodies to connexins, and molecular probes for gap junction messages have shown that most of the normal cells of solid tissues have gap junctions. A few cell types do not, such as normal stem cells (Chang et al, 1987; 1990) or mature red blood cells (although they appear in the early maturation process; Dainiak, 1991; Rosendaal et al, 1991).

Most, If Not All, Cancer Cells Have Dysfunctional GJIC

Clearly, not all cancer cells have been examined. However, very early in the study of gap junctions, Loewenstein (1966) noticed a paucity of GJIC in cancer cells compared to normal cells. While exceptions have been noted (Larsen, 1983), because of the nature of the studies these exceptions do not constitute rigorous negation of the hypothesis. For example, detection of morphological presence of gap junctions does not prove they are functional; some cancer cells do communicate homologously, but not heterologously (Yamasaki et al, 1987); some cancer cells may transfer ions used to measure GJIC, but may not have the cellular signal to suppress growth, etc. Moreover, many studies have indicated a clear decrease or down-regulation of GJIC in various cancer cells (Kanno, 1985).

Most, If Not All, Endogenous and Exogenous Tumor-Promoting Chemicals Reversibly Inhibit GJIC

In the experimental protocol of initiation, promotion, and progression of carcinogenesis in animals, the promotion phase, operationally, is a potentially interruptible or reversible step. In *in vitro* and *in vivo* experiments with endogenous hormones, growth factors and exogenous tumor-promoting chemicals (ie, 12-O-tetradecanoylphorbol-13-acetate, TPA; polybrominated biphenyls, PBBs; phenobarbital; etc; Trosko & Chang, 1988), gap junctional communication can be reversibly down-regulated, either at the posttranslational levels (eg, phosphorylation of GJ protein) or transcriptional level (Bennett et al, 1991). One known tumor promoter, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was claimed to be a noninhibitor of GJIC (Lincoln et al, 1987). However, it has not been demonstrated that TCDD biologically affected those cells or if those cells had receptors for TCDD. Even physical promotion, such as tissue necrosis, cytotox-

icity, or wounding, has been associated with the down-regulation of gap junctions (Yancey et al, 1979; Traub et al, 1983).

Certain Oncogenes Down-Regulate GJIC in a Dose-Dependent Fashion

If tumor-promoting chemicals, which act as growth factors for initiated cells, and growth factors, which can be tumor promoters, inhibit GJIC, then oncogenes, which code for growth factors, growth factor receptors, or transmembrane mitogenic signaling elements, should be able to down-regulate GJIC either transiently (proto-oncogenes) or stably (activated oncogenes) when they transform cells. To date, a series of transforming activated oncogenes, eg, *src*, *ras*, *neu*, *raf*, *mos*, have been shown to down-regulate GJIC in various cell types and dose-dependent fashion (Azarnia et al, 1988; Atkinson et al, 1981; Atkinson et al, 1986; Azarnia & Loewenstein, 1984; Azarnia & Loewenstein, 1987; Chang et al, 1985; Dotto et al, 1989; El-Fouly et al, 1989; Bignami et al, 1988; El-Fouly et al, 1988; Kalimi et al, 1992). In several cell systems, eg, rat liver epithelial oval cells, the oncogenes *ras*, *raf*, and *neu* significantly reduced GJIC; however, together with an expressed *myc*, GJIC was reduced even more (Kalimi et al, *in press*). In addition, the oncogene *ras*, when either linked to a metallo-thionein promoter or exposed to TPA, down-regulated communication either in a direct relationship to the p21 protein produced or synergized with TPA to activate protein kinase-C to down-regulate GJIC completely (Oh et al, 1991; Brissette et al, 1991; de Feijter et al, 1992).

Several Tumor Suppressor Genes Have Been Shown to Up-Regulate GJIC

If the current paradigm is correct that a balance (yin-yang) between oncogene and tumor suppressor genes exists in normal, GJIC cells, then one can surmise that in a nontransformed cell that has an activated oncogene in it a tumor suppressor gene must have negated or ameliorated its effect on the down-regulation of gap junctions.

Lee et al (1991) and Kalimi et al (1990) have recently shown that two potential tumor suppressor genes appear to be correlated with the up-regulation of GJIC, or the prevention of a down-regulation of GJIC. Since tumor-promoting chemicals, activated oncogenes, and growth factors that down-regulate GJIC do so through many biochemical mechanisms (Spray et al, 1988), one can imagine there will be several ways to ameliorate or prevent such down-regulation. For example, if TPA and *ras* affect GJIC by their ability to phosphorylate the GJ protein through protein kinase-C, then dephosphorylation activated by tumor suppressor genes might be one mechanism through which a particular tumor suppressor gene could act. Other examples are in the next two sections.

Anti-Tumor Promoters Such as Retinoids and C-AMP Can Up-Regulate GJIC

Again using the experimental protocol of initiation/promotion/progression in mouse skin, it has been shown that various retinoids could block the tumor-promoting action of TPA (Verma et al., 1979). If the cellular mechanism by which TPA acts as a tumor promoter is via its ability to down-regulate GJIC, then one would predict inhibition of GJIC by TPA should be prevented or ameliorated by the action of retinoids. The results of experiments in an *in vitro* transformation system appear to be consistent with the prediction, in that retinoids prevented the TPA reduction of GJIC (Mehta et al., 1989; Mehta & Loewenstein, 1991).

Recently, Rivedal and Sanner (1992) showed that retinoids, which can promote transformation in Syrian hamster embryo cells in contrast with its ability to inhibit the induction of transformation of C3H10T1/2 and BALB/C 3T3, down-regulate GJIC. In all three cell types, enhancement of communication by retinoids was related to reduced transformation, whereas inhibition of communication was related to enhanced induction of transformation.

Several *in vitro*, as well as *in vivo*, experiments have also shown that increased levels of intracellular C-AMP are associated with enhanced GJIC (Flagg-Newton et al., 1981; Demaziere & Scheuerman, 1985). In initiation/promotion/progression experiments one would then predict increases in C-AMP ought to prevent the promotion of tumors.

Antioncogenic Drugs Such as Lovastatin Reverse RAS-Down-Regulation of GJIC

As understanding of how each oncogene acts to down-regulate GJIC grows, it might be possible to interfere with its mechanism of action. One oncogene, the *H-ras* appears to be functional only after the p21 oncoprotein is bound to the under surface of the plasma membrane. It has been shown that lovastatin can prevent that process (Casey et al., 1989). Interestingly, it also prevents the growth of *ras*-transformed cells in nude mice (Sebti et al., 1991). Recently, it has been shown that lovastatin prevents the *ras*-down-regulation of GJIC in a reversible fashion (Ruch et al., 1993). If a cell has genes that perform the same function in *H-ras* expressed cells, these genes would be considered tumor suppressor genes.

Gap Junction Gene Transfection of GJIC-Deficient and Tumorigenic Cells Restores Their Ability to Communicate

Most assuredly, the ultimate test of the hypothesis would be the restoration of GJ expression in a GJIC-deficient and tumorigenic cell and the subsequent res-

toration of GJIC and reduction of tumorigenicity. Several recent attempts have been successful in the restoration of GJIC in noncommunication tumorigenic cells (Eghbali et al., 1991; Fishman et al., 1991; Mehta et al., 1991; Zhu et al., 1991). Whether these recommunicating tumor cells retain their tumorigenic properties awaits further experimentation.

Gap Junction Antisense Gene Transfected GJIC-Proficient Normal Rat Liver Cells Decreases GJIC

The complementary experiment to the one described above that should test the hypothesis is to transfect an expressible gap junction antisense gene into a GJIC-proficient and normal cell to convert it to a noncommunicating cell and to determine if it is tumorigenic. The authors are currently attempting to express the antisense connexin 43 gap junction gene in normal, GJIC-proficient rat liver epithelial cells. These cells when placed in the nude mice would be predicted to give rise to tumors.

GJIC-Deficient Mutants Derived from Nontumorigenic Rat Liver Cells are Tumorigenic

Several GJIC-deficient mutants have been isolated from the immortal but nontumorigenic GJIC-proficient rat liver WB cell line (Tsao et al., 1984). These mutants have been shown to be defective in posttranslational phosphorylation of the C43 gap junction protein (Oh et al., *in press*). One of these mutants, *ab-1*, has been inoculated into rat livers through portal vein injection for tumorigenicity tests. Liver tumors developed in 10 of 12 rats injected with *ab-1* cells in 10 weeks. The results were repeated in two different laboratories (J. Klaunig of the University of Indiana and C. Welsch of Michigan State University). Compared to *neu* oncogene transfectants that developed liver tumors in three weeks, it took a longer time for the *ab-1* cells to develop the tumors, indicating that GJIC deficiency by itself might be necessary, but insufficient, for triggering the mitogenic process leading to the clonal expansion of tumorigenic transformants in a cell population. In other words, chemical promoters, oncogenes, or growth factors, which act as mitogens for initiated cells, probably can trigger, in a coordinate way, both the down-regulation of GJIC (needed to inhibit contact inhibition) and the onset of the DNA synthesis/mitogenic process. A mutation in a gene that affects only the gap junction structure or function, in and of itself, would not be sufficient to initiate the mitogenic synthetic mechanisms.

SUMMARY

Given that a multicellular organism must regulate its functions of cell proliferation, differentiation, wound healing, and adaptive responses through a homeos-

tatic-cybernetic system, a hypothesis has been advanced, based on the assumption that an integrated extra-, intra- and intercellular communication system exists within and between cells of different tissues, that stable interference of gap junctional intercellular communication, which could occur in a single initiated (nonterminally differentiated) stem or progenitor cell, would lead to cancer. Several tests of the theory were reviewed and all, with varying degrees of rigor, seem to be consistent with the hypothesis. Since gap junctional intercellular communication exists in all organs and tissues, and since it plays a role not only in the regulation of cell proliferation of premitotic cells, eg, its potential role in cancer, blockage of GJIC probably plays roles in many other disease states, for example, teratogenesis (Trosko et al, 1982; Warner et al, 1984); neurotoxicity (Naus et al, 1991; Trosko et al, 1987); reproductive dysfunction (Ye et al, 1990); cardiovascular diseases (Radu & Moldovan, 1991; Smith et al, 1991); cataract formation (Tanaka et al, 1990); and cholestasis (Traub et al, 1983).

In the words of Theodosius Dobzhansky (1973), "Nothing in biology makes sense except in the light of evolution" (p. 125). The gap junction, having shown up in the evolution of metazoans and having been associated with the regulation of cell proliferation and differentiation, has been highly conserved. Any genetic alteration affecting its normal function would block its ability to regulate growth and differentiation. Cancer seems to be the result of such alterations in the somatic tissue, which has been biologically prohibited in the germline since a dysfunctional gap junction expressed after conception would, no doubt, be a lethal event.

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DERMATOTOXICOLOGY

Fifth Edition

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Chapter Twelve

MOLECULAR BASIS OF ALLERGIC CONTACT DERMATITIS

Jean-Pierre Lepoittevin and Valérie Berl

INTRODUCTION

Among the pathological conditions in which chemistry plays an especially important role is, without doubt, contact allergy. Chemical reactions and interactions are involved throughout the biological process that will result in the patient developing delayed hypersensitivity, whether it be during the crossing of the cutaneous barrier (mainly controlled by the physicochemical properties of the allergen), during the formation of the hapten-protein complex (in which chemical bonds are involved), or during the phenomena of recognition between the antigen and the receptors on T lymphocytes (involving a discipline undergoing rapid development, that of supramolecular chemistry).

Recently, there has been a major step forward in our understanding of the molecular basis of hapten recognition by T cells. Nevertheless, this does not eliminate the need to understand the characteristics of the preceding processes, as it is true to say that the properties of a chemical are implicit in its molecular structure.

To cause sensitization, a compound has to penetrate the skin (Potts and Guy, 1992), where it may be metabolized (Hotchkiss, 1992), and react with Langerhans cell surface proteins to form new chemical structures that are recognized as foreign. We discuss in this chapter the way low-molecular-weight chemicals react with skin proteins to form complete antigens and how these structures are recognized by T-cell receptors.

SOME CHEMICAL REMINDERS

Haptens (small molecules with a molecular mass less than 1000 Da) interact with biological macromolecules by mechanisms leading to the formation of bonds of various strengths between the two entities. These bonds, known as chemical bonds, are the result of electronic interactions between atoms and are characterized by the energy that they bring into play, a reflection of their stability. This energy is what must be provided to break the bond between the two atoms. In general, a distinction is made between weak interactions, involving energy levels from a few calories to around 12 kcal/mol of complex, and strong interactions, covalent or coordinate bonds, with bond energies ranging from 50 to 100 kcal/mol.

Weak Interactions

Weak interactions are normally grouped into three main categories: hydrophobic bonds, dipolar bonds, and certain ionic bonds. Although these weak interactions involve modest energy levels

and produce complexes of low stability, they are nonetheless of great biological importance, as they control virtually all the phenomena of recognition between receptors and substrates.

Hydrophobic bonds represent the capacity of organic molecules to organize themselves in water so as to minimize the contact area that they expose to the aqueous solvent. It is by such means that hydrophobic molecules insert themselves into the phospholipid bilayers of cell membranes and into the hydrophobic regions of proteins or membrane receptors. These hydrophobic bonds, which involve energies of the order of 10–20 cal/A²/mol, seem, nevertheless, to play an important role in allergies to very lipophilic products (Darley et al., 1977), such as the allergens from poison ivy (*Rhus radicans* L.) or poison oak (*Rhus diversiloba* T.). This could also be of importance for the interactions of haptens with the lipophilic domains of antigen-presenting cells.

Dipolar bonds are electrostatic interactions between preexisting or induced dipoles. In actual fact, the electron clouds do not always have a uniform charge density (these variations result from the structure of the molecule), and the zones of high electron density can interact electrostatically with zones of low electron density (permanent dipoles). Electron clouds can also be deformed and polarized as they approach one another, thus creating induced dipoles. The interaction between these dipoles is known as van der Waals bonding, with energies of the order of 50–500 cal/mol. Hydrogen bonds are a special case of dipolar interaction. They occur between a hydrogen atom, bound to an electron-attracting atom, and an electron-rich atom. The energy of these bonds can be as high as 5 kcal/mol.

Ionic bonds are electrostatic interactions between preexisting and generally localized charges on organic molecules or minerals. Such interactions occur, for example, between the charged amino acids in proteins and are therefore important in recognition phenomena.

Strong Interactions

Strong interactions, mainly covalent bonds, result when two atoms share a pair of electrons, and are classically represented in chemical formulas by dashes. They involve energies of the order of 50–100 kcal/mol and are therefore very stable compared with the weak interactions. The two electrons required for bond formation can be contributed by both partners, which is called a radical reaction, or can be provided by one of the atoms, which is especially electron rich, and shared with the electron-poor atom; this case is referred to as a reaction between a nucleophile (electron rich) and an electrophile (electron poor). These two terms, nucleophile and electrophile, represent the capacity of a molecule, or rather an atom of this molecule, to donate or accept electrons to form a bond. Nucleophilic centers are rich in electrons and therefore partially negatively charged, while electrophilic centers, deficient in electrons, are partially positively charged.

Mechanisms of Bond Formation

The main mechanisms for the formation of covalent bonds involved in contact allergy can be grouped into three main categories: nucleophilic substitutions, on either a saturated or unsaturated center, and nucleophilic additions.

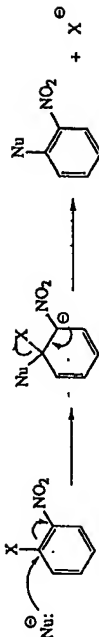
Nucleophilic substitution on a saturated center (Figure 1) involves the attack by an electron-rich nucleophile on an electron-poor electrophilic center. As the electrophilic carbon already has four single bonds, a new bond can only be formed if one of the existing bonds is broken. The overall effect will therefore be a substitution of one of the groups (the leaving group) by the nucleophile.

A nucleophilic substitution reaction can also take place at an unsaturated center (a carbon with one or more multiple bonds). In this case, although the overall result is again a substitution, the mechanism is slightly different. The presence of a multiple bond allows the formation of a saturated intermediate and the subsequent reformation of the multiple bond, permitted by the departure of the leaving group, resulting in the substitution product. This mechanism is

- Nucleophilic substitution on a saturated center



- Nucleophilic substitution on an unsaturated center



- Nucleophilic addition



Figure 1. Principal mechanisms of covalent bond formation seen in contact allergy.

illustrated in the aromatic series in which it is all the more favored by attracting groups (e.g. nitro), which stabilize the intermediate.

Nucleophilic addition is simply the addition (with no leaving group) of a nucleophilic atom to an unsaturated electrophilic center (containing one or more multiple bonds). This mechanism is very similar to the first stage of nucleophilic substitution on an unsaturated center, but the absence of a leaving group rules out the reformation of the multiple bond. A saturated compound is thus produced.

Coordination Bonds

Another type of relatively strong bond, comparable to covalent bonds, is found; this occurs between metals or metal salts and electron-rich atoms (mainly heteroatoms, such as nitrogen or oxygen). These interactions, known as coordinate bonds, permit these electron-rich groups (the ligands) to transfer part of their electron density to the metal and increase its stability. Coordinate bonds are characterized by the number of ligands and by a geometry characteristic both of the metal and of its degree of oxidation (Figure 2). For example, cobalt(II) (Co²⁺) is

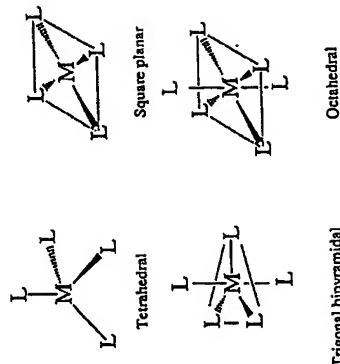


Figure 2. Examples of coordination geometry.

characterized by a tetrahedral arrangement, nickel(II) (Ni^{2+}) by a square planar tetra coordinated arrangement, and chromium(III) (Cr^{3+}) by a six-ligand octahedral arrangement. The number of ligands and the geometry of these coordination complexes determine whether the metals are allergenic and control cross-reactions.

PRINCIPAL ELECTROPHILIC CHEMICAL GROUPS PRESENT IN CONTACT ALLERGENS

Many chemical groups have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. Table 1 shows those chemical groups most frequently found in contact allergens and the mechanism by which they react with nucleophilic groups. The previously defined three main types of mechanism, nucleophilic substitution on a saturated center (e.g., alkyl halides and epoxides), nucleophilic substitution on an unsaturated center (aromatic halides or esters), and nucleophilic addition (carbonyl derivatives and α , β -unsaturated systems), can be seen.

BACK TO CONTACT ALLERGY

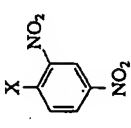
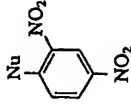



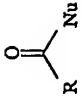


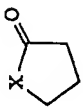
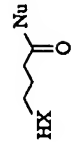

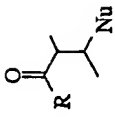

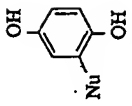

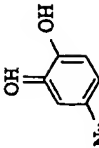
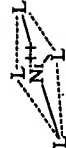
If we consider the human body in its entirety and from a chemical viewpoint, it becomes apparent that a very large proportion of biological structures, especially nucleic acids and proteins, contain many electron-rich groups (those containing nitrogen, phosphorus, oxygen, or sulfur). We can thus consider the human body as being overall nucleophilic. It is therefore not surprising that many biological mechanisms are disturbed on contact with electrophilic chemical substances. Depending on the site of action of these electrophilic molecules, the effect can be mutagenic (Frierson et al., 1985), toxic (Guengerich and Liebler, 1985), or allergenic if the target is the skin. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens (Figure 3). Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can react with electrophiles (Means and Feeney, 1971). Thus it has been shown by nuclear magnetic resonance (NMR) that nickel sulfate was interacting with histidine residues of peptides bound to a major histocompatibility complex (MHC) molecule (Romagnoli et al., 1991) and that methyl alkane sulfonates, allergenic methylating agents, were mainly reacting with histidine and to a less extent with lysine, methionine, cysteine, and tyrosine (Lepoittevin and Benzeva, 1992). If we consider the chemical structure of some allergens (Figure 4) in the light of the chemical principles already outlined, it is easy to understand that all of these molecules will be able to react with biological nucleophiles, certain amino acids in proteins, to form extremely stable covalent bonds and thus lead to the triggering of delayed hypersensitivity. Again, the previously described three main types of mechanism for the formation of covalent bonds are seen; the arrows indicate the reactive center of each molecule.

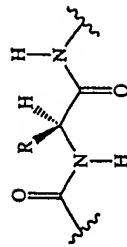
In recent years, the radical mechanism has gained increased interest in the discussion of the mechanism of hapten-protein binding (Schmidt et al., 1990). This mechanism, which has never been firmly established, has been postulated to explain, for example, the allergenic potential of eugenol versus *iso*-eugenol (Barat and Basketter, 1992). More recently, studies indicating that radical reactions were important for haptens containing allylic hydroperoxide groups have been published (Gäfvert et al., 1994; Lepoittevin and Karlberg, 1994).

THE HAPTEN-PROTEIN BOND: COVALENT OR NONCOVALENT?

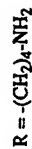
In biology, few phenomena are irreversible, with the majority being controlled by equilibria. It is easy to understand that the more stable the hapten-protein complex, the greater is the possibility of the immune system being able to process the immunological information, resulting in allergy. Given this, we can understand why the very strong and difficult-to-reverse covalent

Table 1. Principal electrophilic groups seen in contact allergy, with mechanisms of reaction with nucleophiles and the products

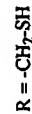
Group	Name	Reaction mechanism	Product
$R-CH_2-X$ $X = Cl, Br, I$	Alkyl halide	Nucleophilic substitution on a saturated center	$Nu-CH_2-R$
	Aryl halide	Nucleophilic substitution on an unsaturated center	
$X = F, Cl, Br, I$			
	Aldehyde; $R' = H$ Ketone; $R' = \text{alkyl or aryl}$	Nucleophilic addition	
	Ester; $R' = OR$ Amide; $R' = NHR$	Nucleophilic substitution on an unsaturated center	
	Epoxide	Nucleophilic substitution on a saturated center	
	Lactone; $X = O$ Lactam; $X = NH$	Nucleophilic substitution on an unsaturated center	
	Unsaturated aldehydes and ketones	Nucleophilic addition	
$R = H, R, OR$			
	<i>para</i> -Quinone	Nucleophilic addition	
	<i>ortho</i> -Quinone	Nucleophilic addition	
$Ni^{++}, Co^{++}, Cr^{IV}$	Metal salts	Coordination bonds	



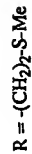
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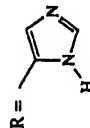
Cysteine



Methionine



Histidine



Tryptophane

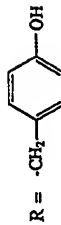
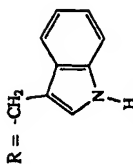


Figure 3. Principle nucleophilic residues in proteins.

bond produces a maximal biological efficacy. It is therefore natural that it is this type of bond that is found in the majority of cases of allergy. However, it would be incorrect to think that only covalent bonds result in allergy. In the case of metal salts, the formation of a covalent bond is impossible, and it is clear that a sufficiently stable coordination complex must be formed between the metal salt and the electron-rich residues of proteins (Polak and Frey, 1973; Hutchinson et al., 1975). These coordination complexes are therefore sufficiently stable, and the protein modification sufficiently important, to lead to allergy (Sinigaglia, 1994).

METABOLISM AND PROHAPTENS

Far from being an inert tissue, the skin is the site of many metabolic processes, which can result in structural modification of xenobiotics that penetrate into it. These metabolic processes, primarily intended for the elimination of foreign molecules during detoxification, can, in certain cases, convert harmless molecules into derivatives with electrophilic, and therefore allergenic, properties. The metabolic processes are mainly based on oxidation reactions via extremely powerful enzymatic hydroxylation systems, such as the cytochrome P-450 enzymes (Mansuy, 1985), but monoamine oxidases, which convert amines to aldehydes, and peroxidases seem to play an important role in the metabolism of haptens. When activated by the production of hydrogen peroxide during the oxidative stress following the introduction of a xenobiotic into the skin, peroxidases convert the electron-rich aromatic derivatives (aminated or hydroxylated) into quinones, which are powerful electrophiles. In this way, the long-chain catechols, responsible for the severe allergies to poison ivy (*Rhus radicans* L.) and poison oak (*Rhus diversiloba* T.), are oxidized *in vivo* to the highly reactive orthoquinones (Dupuis, 1979) (Figure 5). The same applies to paraphenylenediamine or hydroquinone derivatives, such as the allergens from

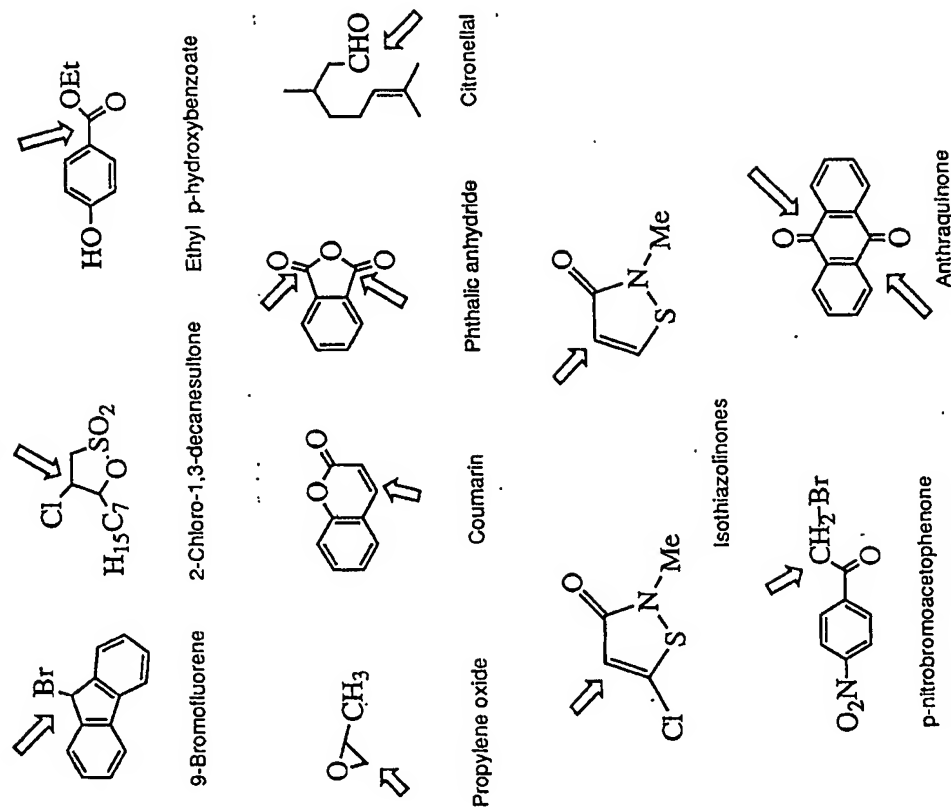


Figure 4. Examples of allergizing molecules. The electrophilic center is indicated by an arrow.

Phacelia crenulata Torr. (Reynolds and Rodriguez, 1981), which are converted into electrophilic paraquinones. Metabolic reactions involving enzymatic hydrolyses can also occur in the skin. It is thus that the tuliposides A and B, found in the bulb of the tulip (*Tulipa gesneriana* L.), are hydrolyzed, releasing the actual allergens, tulipalines A and B (Bergmann et al., 1967).

All these molecules, which have themselves no electrophilic properties and cannot therefore be haptens but which can be metabolized to haptens, are referred to as prohaptens (Landsteiner and Jacobs, 1936; Dupuis and Benzra, 1982) and play an important role in contact allergy because of their number and their highly reactive nature. The fact that the structure of the metabolized molecule can be far removed from the structure of the initial molecule can make allergologic investigations even more difficult.

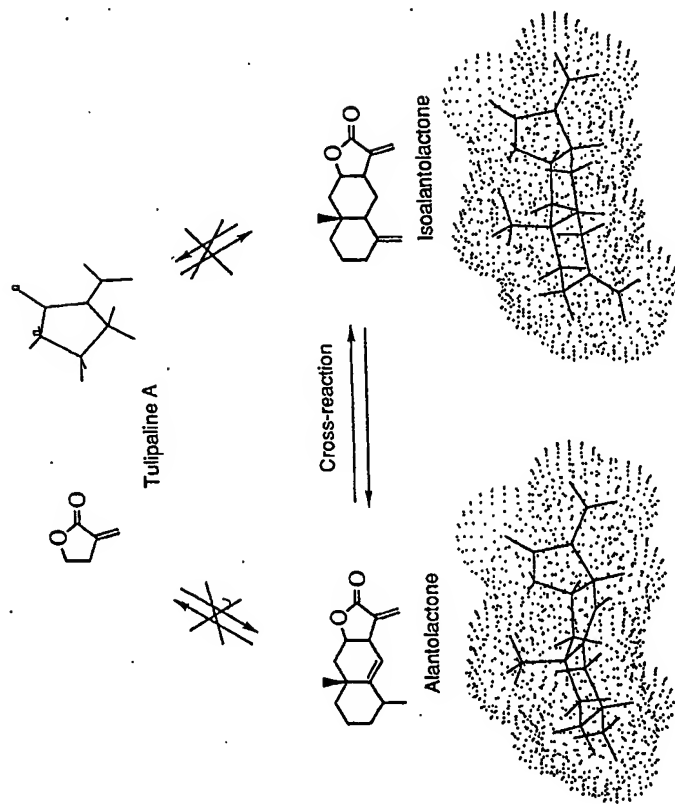


Figure 7. Chemical structure and spatial representation of tulipaline A, alantolactone, and isovalantolactone.

The identification of cross-allergenic responses can be especially difficult, particularly in humans, in whom the possibility of co- or polysensitization should never be ruled out. In addition, the metabolism of molecules can be very complex, and two molecules with a priori little in common can be converted to derivatives that have a similar structure. Thus, derivatives of hydroquinones and *para*-phenylenediamines can be converted into benzoquinone derivatives. It is therefore dangerous to draw conclusions from tests without knowing how the substances are, without doubt, due to co-sensitization (Benezra and Maibach, 1984). Experimental studies in animals are often the only means of being really certain of what happens during recognition. The concept of the prohaptens is very important in the interpretation of results in allergy. As the structure of the metabolized molecule can sometimes be very different from that of the initial molecule, it can be difficult to establish similarities of chemical groups and structure.

Molecular Modeling as a Tool for Cross-Reactivity Analysis

In the last few years, molecular modeling has been shown to be a powerful tool in studies of conformation-dependent drug-receptor interactions and structure-activity relationship analysis (Cohen et al., 1990). Despite the great potential of this technique, few attempts to analyze cross-reaction patterns in the field of allergic contact dermatitis have yet been reported. One reason may be the heterogeneous population of patients with heterogeneous clinical histories, in which it is somewhat difficult to distinguish between actual cross-reaction and concomitant sensitization. A second reason is that, to be effective, structure-activity relationship studies

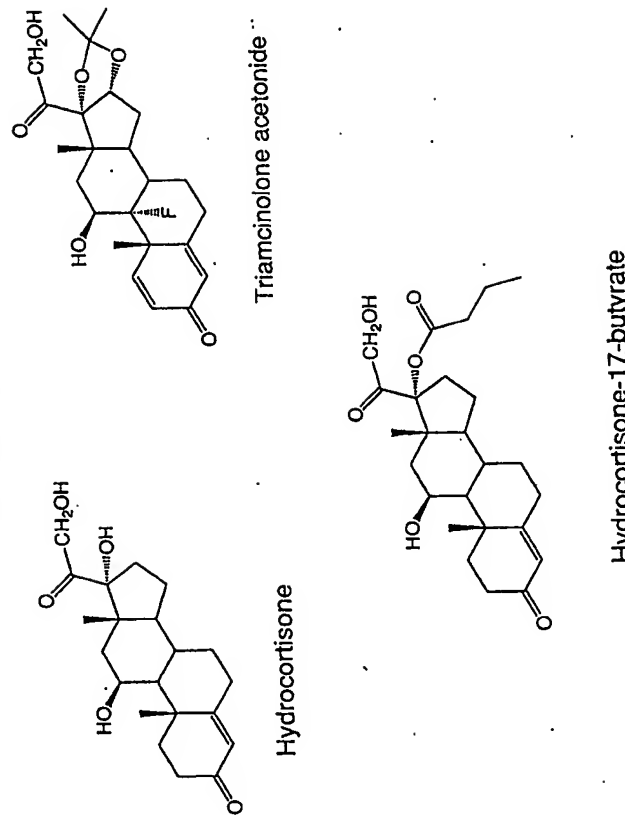


Figure 8. Chemical structure of hydrocortisone (class A), triamcinolone acetonide (class B), and hydrocortisone-17-butyrate (class D).

need data for a wide range of molecules. The clinical investigation of contact dermatitis to corticosteroids, in which a large number of related substances are tested on a large number of patients, represents a good opportunity to carry out such a structure-activity study. From the statistical analysis of the clinical data, it is now possible to advance an experimentally supported hypothesis for cross-reaction patterns. Coopman et al. (1989) hypothesized that cross-reactions occur primarily within certain groups of corticosteroids. They distinguished four groups, group A consisting of hydrocortisone, tixocortol pivalate, and related compounds, group B consisting of triamcinolone acetonide, amcinonide, and related compounds, group C consisting of betamethasone, dexamethasone, and related compounds, and group D consisting of esters such as hydrocortisone-17-butyrate and clobetasone-17-butyrate (Figure 8). It is now possible to correlate this with conformational characteristics and to establish a molecular basis for cross-reaction patterns in patient sensitized to corticosteroids. This could be invaluable in the prediction of potential cross-reactions to new molecules.

Example of Conformational Analysis: Cross-Reaction to Corticosteroids

The conformation of corticosteroids from groups A, B, C, and D was analyzed (Lepoittevin et al., 1994). This study was based on two hypotheses. The first was that all corticosteroids should interact with proteins in a very similar way. All corticosteroid molecules were designed to interact with the same type of receptors, and thus should be more or less metabolized in similar ways. The second hypothesis, based on chemical observations, was that esters at position 21 are readily hydrolyzed to give the free alcohol while esters at position 17 are more resistant to hydrolysis, due to a strong steric hindrance. Thus, for example, tixocortol pivalate was

considered as tixocortol with a free thiol group at position 21, and alclometasone 17,21-dipropionate was considered as alclometasone 17-propionate.

All molecules were drawn from energy-minimized building blocks and were then submitted to a multiconformational analysis in order to achieve the most energetically stable conformation. These conformations were then compared for analogies or differences in the van der Waals volumes that define the electronic shape of the molecule. As expected from the hypothesis, significant group-specific characteristics of volume and shape were found for molecules of group A, B, and D but not for molecules of group C.

In terms of molecular characteristics, the existence of groups A, B, and D, as defined by the analysis of cross-reaction patterns in patients sensitized to corticosteroids, is fully supported by the conformational analysis of these molecules. Molecules of the same group have very similar spatial structures, explaining the cross-reactions observed. In addition, molecules from one group are sufficiently different from molecules of another group to explain the lack of cross-reactions observed between groups A, B, and D.

The volume occupied by specific groups on the α face of ring D seems to be critical for the molecular recognition of corticosteroids by receptors of immunocompetent cells, while modifications of other parts of the molecule seem to have little effects on the recognition patterns. As shown in Figure 9, each group represents a well-defined, characteristic shape that can be very useful for the prediction of potential cross-reactions of new corticosteroid molecules.

CONCLUSION

The principles that we have just discussed permit a rational approach to the phenomena of contact allergy, but, in actual fact, we often have available only indirect evidence suggestive of one mechanism or another. Although the chemical bases for hapten-protein interactions can be checked in the laboratory by the use of nucleophilic amino acids, small peptides, and model proteins, and although a certain number of steps can be checked, at the present time, no method is available to follow a hapten step by step during the entire immunological process leading to contact allergy. Many points await investigation, but in many cases a "chemical" analysis of the problem does allow us to understand and to foresee cross-allergies and thus to warn the patient about structurally related products.

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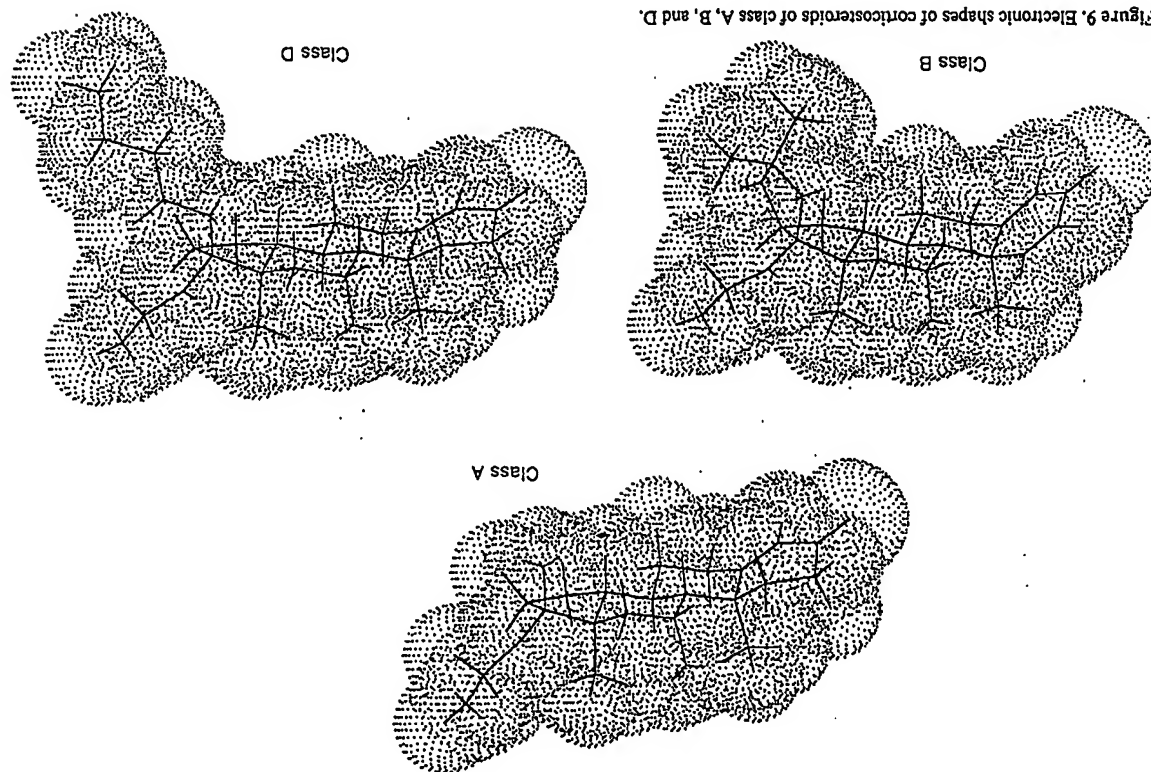


Figure 9. Electronic shapes of corticosteroids of class A, B, and D.

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Chapter Thirteen

SYSTEMIC CONTACT-TYPE DERMATITIS

Torkil Menné, Niels Veien, and Howard I. Maibach

INTRODUCTION

Systemic contact dermatitis is an inflammatory skin disease that may occur in contact-sensitized individuals when these persons are exposed to the hapten orally, transcutaneously, intravenously, or by inhalation. The entity can be present with clinically characteristic features or be clinically indistinguishable from other types of contact dermatitis. Contact sensitization to ubiquitous haptens is common. In a recent Danish population-based study, 15.2% reacted to one or more of the haptens in the Standard patch test series (Nielsen and Menné, 1992). The total number of individuals at risk of developing a systemic contact dermatitis reaction is therefore large. While systemic contact dermatitis reaction from medications is a well-established entity, systemic reaction from metals, particularly nickel, is still regarded as controversial (Burrows, 1992; Möller, 1993).

The first description of systemic contact dermatitis can probably be ascribed to the pioneering British dermatologist Thomas Bateman (Shelley and Crissey, 1970). His description of the mercury eczema called *eczema rubrum* is similar to what we today describe as the baboon syndrome:

Eczema rubrum is preceded by a sense of stiffness, burning, heat and itching in the part where it commences, most frequently the upper and inner surface of the thighs and about the scrotum in men, but sometimes it appears first in the groins, axillae or in the bends of the arms, on the wrists and hands or on the neck.

In this century the systemic spread of nickel dermatitis was described by Schittenhelm and Stockinger in Kiel in 1925. By patch testing nickel-sensitive workers with nickel sulfate, they observed the spread of dermatitis and flares in the original areas of contact dermatitis. The literature on systemic contact dermatitis is now comprehensive. Recent reviews include Cronin (1980), Fisher (1986), and Veien et al. (1990).

CLINICAL FEATURES

The clinical symptoms related to systemic contact dermatitis are summarized in Table 1. The symptoms may appear exclusively on the skin, but general symptoms are occasionally seen. Knowledge of the clinical symptoms stems from clinical observations and experimental oral challenge studies.

Flare-up reactions in the primary site of dermatitis or previously positive patch-test sites raise the suspicion of systemic contact dermatitis (Ekelund and Möller, 1969; Christensen and Möller, 1975; Menné and Weismann, 1984). Flare-up of previously positive patch-test sites following ingestion of the hapten is a fascinating and specific sign of a systemic contact

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